

Liquid Chromatography: Theory and Methodology

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INTRODUCTION

This review covers fundamental developments in liquid chromatography during the period of approximately December 1989 through December 1991. Again this year there are separate reviews on instrumentation and size exclusion chromatography; this review is of important developments in the chemistry of the separation process. The primary searching methods for this work have been CAS Online and CA Selects. Each author has supplemented these with search methods of their own.

This is not meant to be a comprehensive review of all published papers during this time period; rather, we have tried to select those papers which we feel are significant developments. We have largely restricted the covered material to the readily accessible English language literature. Comments and suggestions concerning this review are welcomed and should be sent to the first author (J.G.D.).

A. BOOKS, REVIEWS, AND SYMPOSIA PROCEEDINGS

Where possible in this section, reviews are cited along with published books. Unger edited a volume titled *Packings and Stationary Phases in Chromatographic Techniques* (A1) which was reviewed by Evans (A2) and Sander (A3). Ahuja published a book titled *Selectivity and Detectability Optimization in HPLC* (A4) which was reviewed by Glajch (A5) and by Smith (A6). Glajch and Snyder edited a volume titled *Computer Assisted Method Development for High Performance Liquid Chromatography* (A7) which was reviewed by Warren (A8), and the 30th volume of the popular *Advances in Chromatography* series appeared (A9) and was reviewed (A10). Many other more specialized books appeared during this review period; where appropriate they are referenced in the individual sections later in this review.

Brown discussed the history and future of modern liquid chromatography (LC), especially as related to the separations of nucleosides and related separations (A11). Majors published a two part review discussing new LC columns introduced at the 1991 Pittsburgh Conference (A12, A13).

Meyer published a perspective on the First International Symposium on Column Liquid Chromatography (A14) held in 1973. This has become more commonly known as HPLC'xx, and both the 13th and 14th of these yearly meetings were held, and the proceedings published (A15, A16).

B. THEORY AND OPTIMIZATION

Theory

With the exception of Giddings' unifying monograph on

separation science (B1) and the reviews cited immediately below, we have excluded nearly all theoretical contributions that can logically be placed in another category elsewhere in this review (e.g., reversed phase, geometric and optical isomers, preparative, etc.), especially those contributions pertaining to chromatographic properties (efficiency, retention and selectivity).

Reviews. Sumpter and Lee provided an elegant review of the methods for enhancing radial dispersion in open tubular column chromatography, including turbulent and secondary flow (B1A). Small gave a historical account of ion chromatography over the last 20 years (B2). Snyder reviewed the gradient elution separation of large biomolecules (B3), with an emphasis on peptides and proteins. Treatises on (i) the solutions of the equilibrium and semiequilibrium models and linear and nonlinear chromatography (B4) and (ii) the influence of the choice of boundary conditions on the solutions to the dynamic (kinetic) chromatography models (B5) were presented by Guiochon and co-authors. Finally, Foley reviewed the concept of resolution in linear column chromatography and compared the accuracy and simplicity of two new expressions with those introduced previously (B6).

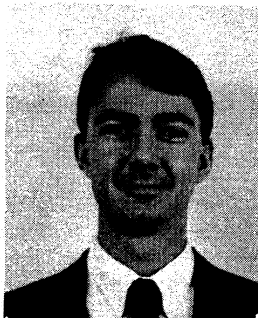
General. Scott examined the design of liquid chromatography capillary columns and presented relationships for the calculation of the column length, radius, and film thickness corresponding to the fastest separation (B7); for critical pair separations, the capacity factor (k') of the first solute should be 2.7. Tock et al. examined open tubular columns from the perspective of mass loadability and showed that better results will be obtained if the contribution to total plate height from stationary phase mass transfer is allowed to be nearly 50% instead of the arbitrary 20% ceiling that is typically imposed (B8). Alhedai and co-workers showed that two types of chromatographically important "dead volumes" exist: a kinetic one (volume of moving phase) that is common to all solutes and a thermodynamic one that is unique for each solute due to solute-specific exclusion properties of the stationary phase (B9). Golshan-Shirazi and Guiochon presented a comprehensive theory of system peaks that permits the prediction of response factors in the case when an additive is used, among other things (B10). Valentin used the mathematical concept of zonoids to put chromatography in perspective as a separation process (B11). Finally, Wang and Lung described mass transport for random walks with a log-normal waiting-time probability (B12).

The effect of pressure on chromatographic variables was the subject of two studies. Poe and Martire reported a plate height theory for compressible mobile-phase fluids and its application to gas, liquid, and supercritical fluid chromatography (B13); expressions applicable to gases, liquids, and supercritical fluids were derived in terms of temporal and

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Joe P. Foley is an Associate Professor of Chemistry at Villanova University. He received his B.S. in chemistry and chemical physics from Centre College of Kentucky in 1978 and his Ph.D. in Chemistry at the University of Florida in 1983. He then accepted a 2-year National Research Council Postdoctoral Fellowship at the National Institute of Standards and Technology. In August of 1985, Dr. Foley joined the faculty of Louisiana State University and continued there until accepting his appointment at Villanova in 1991. Dr. Foley's research interests are in the fundamental and applied aspects of chemical separations, and he has published about 30 articles and book chapters pertaining to modern liquid chromatography, supercritical fluid extractions/chromatography, capillary zone electrophoresis, and micellar electrokinetic chromatography. A participant at the recent NATO Advanced Study Institute on Theoretical Advances in Chromatography and Related Separation Techniques, Dr. Foley has organized symposia for the Pittsburgh Conference, the American Chemical Society, and the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS) and has served as a review panelist for the Health Effects Institute and as a consultant for instrument manufacturers and the chemical industry. A former member of the Executive Board of the New Orleans Chromatography and Analytical Discussion Group, Dr. Foley is currently a member of Sigma Xi, the American Chemical Society, and the Executive Committee of the Chromatography Forum of the Delaware Valley. He also serves on the Editorial Boards of the *Journal of Microcolumn Separations* and *The Analyst*.

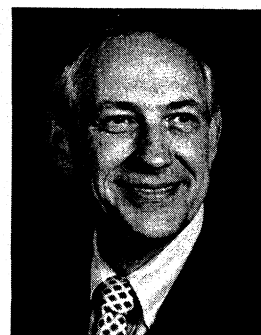


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spatial average values of local plate height, solute capacity factor (k'), and mobile-phase density. McGuffin and Evans studied the influence of pressure on retention and selectivity (B14), and found a surprisingly large pressure-dependence of k' and α for C10-C20 derivatized fatty acids.

Robert A. Barford is Research Leader at the Eastern Regional Research Center of the Agricultural Research Service-United States Department of Agriculture, located in Philadelphia, where he conducts research: (1) to ascertain structures of biopolymers including proteins and polysaccharides, determine factors that perturb these structures and relate the structures to functional, biochemical and nutritional properties of agricultural products; (2) to develop new technologies, in support of USDA's regulatory branch, for expeditiously detecting chemical residues and their metabolites in animal products. A graduate of Temple University, he has authored or coauthored over 120 scientific publications, contributed five book chapters, and coedited the books *Methods for Protein Analysis* and *Interactions of Food Proteins*. He is an active member of the Analytical and the Agricultural and Food Chemistry Divisions of the ACS and the Chromatography Forum of the Delaware Valley. He has organized numerous symposia for these organizations and was Chairman of the Sixth International Symposium on Column Liquid Chromatography and General Chairman of the 1981 Meeting of the Federation of Analytical Chemistry and Applied Spectroscopy Societies. He has received the Chromatography Forum of the Delaware Valley Award and the Pennsylvania Chapter of the American Institute of Chemists Honor Scroll.



Howard G. Barth is a member of the research staff of the Analytical Division of Central Research & Development at Du Pont Experimental Station, Wilmington, DE. Before joining the Du Pont Co. in 1988, he was a research scientist and group leader at Hercules Research Center. He received his B.A. (1969) and Ph.D. (1973) in analytical chemistry from Northeastern University. His specialties include polymer characterization, size-exclusion chromatography, and high-performance liquid chromatography. He has published over 50 papers in these and related areas. Barth has also edited the book *Modern Methods of Particle Size Analysis* (Wiley, 1984) and coedited *Modern Methods of Polymer Characterization* (Wiley, 1991). He has also edited three symposium volumes on polymer characterization published in the *Journal of Applied Polymer Science*. Barth was on the Instrumentation Advisory Panel of *Analytical Chemistry* and was Associate Editor of the *Journal of Applied Polymer Science*. He is cofounder and Chairman of the International Symposium on Polymer Analysis and Characterization. Barth is past Chairman of the Delaware Section of the ACS where he presently serves as councilor. Dr. Barth is a member of the ACS divisions of Analytical Chemistry, Polymer Chemistry, and Polymeric Materials Science and Engineering, Society of Plastics Engineers, and the Delaware Valley Chromatography Forum. He is also a Fellow of the American Institute of Chemists.



Three new methods of elution were introduced. Little et al. reported an inventive method for achieving multidimensional-like separations on a single HPLC column via the sequential application of one or more selective mobile-phase gradients prior to a universal (solvent or micellar) gradient (B15). This sequential multimodal elution facilitated both between-class and within-class separations, by doubling the peak capacity and reducing the separation disorder by ln 2. Wahl and co-workers reported the novel concept of solvent modulation (B16), in which individual solvent zones are introduced onto a column in a varying or repeating sequence. Overall retention can be accurately modeled by the retention in each zone; precision is inversely related to zone width. Finally, Sulya and co-workers developed and evaluated thermal and transient step mobile phase gradient elution for single-pump HPLC systems as an alternative to isocratic elution that facilitates analyte preconcentration (B17).

Velayudhan and Ladisch investigated the influence of mobile-phase additives on gradient shape (B18). Gradients can be significantly deformed (ultimately to shock waves) if the concentration of the additive is beyond the linear portion of the isotherm. Foucault and Nakanishi compared gradient elution in centrifugal partition chromatography with HPLC and found the advantages to be similar (B19). Kawasaki et al. utilized a competitive site mechanism to model the gradient elution behavior of several proteins and nucleoside phosphates on hydroxyapatite stationary phases (B20). Reproducibility problems in gradient elution caused by differing equipment

were considered by both Snyder and Dolan (B20) and MacLeod (B21); the latter proposed isocratic delay volumes of 5 and 0.5 mL for standard and microbore systems, respectively, as a solution to this problem. Finally, two new designs for solvent programming were presented by Berry and co-authors (B22,B23), with the latter geared toward capillary electrophoresis and micro-LC.

Several researchers investigated a variety of phenomena that gave rise to anomalous peak shapes. Hoffman and Rahman used equilibrium distribution theory to approximate band shape for combinations of strong injection solvent and weak mobile phase (B24) and showed that the peak height and plate number of solutes with $k' < 3$ was increased dramatically under certain circumstances. Zapata and Garrido observed a variety of peak shape artifacts in their RPLC separation of chlorophylls and carotenoids that they attributed to solute-injection solvent interactions (B25). Gesquiere et al. showed that slow cis-trans isomerization of some proline-containing peptides produced peak splitting during reversed-phase HPLC but that this problem could be minimized by working at elevated temperatures (B26). Finally, Evans and McGuffin examined refractive index artifacts in absorbance detectors due to nonideal injection and gradient elution and utilized a ray-tracing algorithm to predict the apparent absorbance (B27).

Three noteworthy reports on specific separation modes were published during this review period. Johnston and Hearn compared experimental findings with predictions of several adsorption models for proteins on porous coulombic anion-exchange sorbents and found that the best agreement using the sophisticated model of Arve and Liapus (B28). Assuming that the shapes of proteins can be approximated as cubes, Staahlberg et al. offered an elegant electrostatic interaction model as an alternative to the stoichiometric displacement models to describe the ion-exchange chromatography of proteins (B29); their model is based on the solution to the linearized Poisson-Boltzmann equation for two oppositely charged planar surfaces in contact with a salt solution. Finally, Potschka reported a general theoretical description of size exclusion chromatography based on data obtained using DNA and viruses as probes as well as from critically reviewed data from the literature (B30). Electrostatic forces alone were insufficient to explain the experimental results, particularly for relatively small molecules (<3 nm) where hydration forces were observed to dominate.

Optimization

Reviews. Ahuja discussed the optimization of selectivity, detectability, and analysis time in HPLC from the perspective of pharmaceutical analysis (B31). Brereton provided an overview of chemometric approaches (B32), whereas Berridge focused on the simplex algorithm for the optimization of isocratic and gradient elution (B33). Billiet and De Galan discussed the selection and optimization of mobile-phase parameters in reversed-phase and ion-pairing chromatography (B34). Coenegracht et al. compared methods of optimization in RPLC with emphasis on isoeluotropic or nonisoeluotropic ternary and quaternary mobile phases (B35). Glajch and Kirkland summarized method development in reversed-phase, normal-phase, ion-pair, and gradient elution HPLC, including retention mapping and experimental design techniques, numerical separation criteria, peak tracking, and software (B36). Hanai focused on biomedically important aromatic acids and N-containing compounds in his 17-reference review on advanced optimization techniques (B37). Hayashi and Matsuda reviewed the results obtained by optimizing R_s with those obtained by optimizing a composite function of several chromatographic parameters via information theory and found the latter to be superior (B38). Jandera concentrated on the predictive calculation methods for the optimization of gradient elution using binary and ternary mobile phases (B39). Snyder and co-workers reviewed the features and advantages of computer simulation software for the optimization of isocratic (B40) and gradient elution (B41). Finally, Schoenmakers and Dunand provided explanations and advice provided by an expert system for system optimization in HPLC (B42).

Original Papers. Computer simulations have begun to play an increasingly important role in the optimization of separations and in the understanding of retention phenomena.

Several reports using in-house or commercial packages appeared in this review period, most concerned with pharmaceutical applications. Of the simulations developed in-house, Baba and co-workers used simulations procedures in-house to optimize gradients in the anion-exchange chromatography of oligonucleotides (B43) and inorganic cyclic polyphosphates (B44). Jinno and co-workers reported a system (MCASYST) for the automated qualitative and quantitative analysis of toxic drugs (B45), and Lankmayr and co-authors described a similar one for pharmaceuticals that placed special emphasis on the chemical selectivity of the separation and ruggedness (B46). Heinisch and co-workers employed in-house computer simulations for the optimization of mobile phase composition in gradient elution RPLC (B47), and Markowski et al. employed a similar approach for the separation of some phenolic pollutants by gradient elution HPLC (B48). Wang et al. employed a window diagram approach (B49) and a mixture design simplex method (B50) for the computer-assisted optimization of mobile phase, pH, and/or ion concentration. Geiger and Rimpler reported a program for natural products based on a structured trial and error approach (B51), and Djordjevic et al. reported an HPLC system capable of fully automatic optimization (B52). Last but not least, Lissester wrote and published a PASCAL program for the optimization of ternary mobile phases for RPLC based on a seven point factorial design (B53).

Commercial software packages were also represented during this review period. Snyder and co-workers extended the capabilities of DryLab to include nonlinear gradients (B54) or the simultaneous change of two or more variables (with some restrictions) (B55). Lehmann and Miller applied DryLab to the separation of fluoroxypyr herbicide and metabolites (B56). The trimodular ICOS system described by Faulstich and Catalano consists of three interactive modules for (i) selection of three isoeluotropic mobile phases, (ii) selectivity optimization via a lattice search, and (iii) retention modeling for interpretation (B57). Nyiredy et al. provided a detailed account of the capabilities of the "PRISMA" system, including automatic peak identification (B58), while Outinen et al. described its use for the separation of biogenic amines (B59). Mazerolles and co-authors summarized computer-assisted optimization with NEMROD software (B60), and Mant et al. discussed ProDigest-LC, a method development simulation package specifically designed for peptide separations (B61).

Interest in the nuances of chromatographic response functions (CRFs) has continued. Cela et al. critically evaluated several previously reported CRFs and discussed their role in optimization and method development (B62). Deming and co-authors reported several algorithms and experimental designs for the optimization and interpretation of various user-selected chromatographic and analytical parameters (B63). Bourguignon and Massart investigated Derringer's multicriterion desirability function and compared the results with those achieved using conventional CRFs and other multicriterion functions such as Pareto-optimality (B64). Matsuda and co-workers used information theory to develop (B65) and modify (B66) a CRF they coined "function of mutual information (FUMI)" and demonstrated its advantages when used with the simplex algorithm for the optimization of mobile-phase composition in RPLC (B65, B67). To avoid finding local optima when using the simplex algorithm, Lu and Huang developed a multifactor, hierarchical CRF based on the number of peaks, resolution, analysis time, etc. (B68).

Numerous chemometric approaches to optimization were presented. Hu and Massart utilized the uniform shell (Doehlert matrix) design for RPLC; this 2-factor design requires seven experiments and yields a quadratic model with interaction terms (B69). Dondi et al. employed sequential methods (Fibonacci and simplex) to optimize linear gradient elution in RPLC; results using different organic modifiers were compared and the no. of components was estimated using the Davis-Giddings statistical overlap theory (B70). Conti and co-authors developed a complex expert system for HPLC method development through a computer "supervisor" that decides which of several smaller expert systems to employ at a given stage (B71). Along similar lines Dolan and Snyder proposed the strategic integration of an assortment of computer-aided method development techniques in order to maximize their individual strengths while compensating for their respective weaknesses (B72). Valko and co-workers used

expert systems to predict retention of metabolites (B73) and the initial mobile-phase conditions for selectivity optimization (B74) in pharmaceutical analysis.

A series of papers utilizing information theory was published by Hayashi et al. for the optimization of resolution (B75), flow rate and column length (B76), quantity and/or detection wavelengths (B77, B78), multiple chromatographic variables (B79, B80), degree of separation for purposes of chromatographic peak characterization (B81), and mobile-phase composition for good precision (B82). Solutions to problems involving unresolved peaks were presented in conjunction with QSR (B83).

Several authors utilized retention time prediction and/or response surface mapping as a means of optimizing separations. Dasko employed the UNIFAC group contribution method to predict retention and determine activity coefficients of solutes in the mobile and stationary phases in RPLC (B84). Harmala and co-workers employed molecular connectivity to predict retention in their PRISMA-structured isocratic and gradient elution systems (B85). Hase and Ikenaka described methods for estimating elution times of phnyridylamino derivatives of sugar chains from glycoproteins in RPLC (B86), and Grushka and co-workers reported retention and selectivity surfaces of deoxyribonucleosides in RPLC (B87). Naish-Chamberlain and Lynch utilized empirical piece-wise quadratic modeling (B88), whereas Valko and Slegel employed molecular modeling to predict solute retention and subsequently optimize the mobile phase in RP-HPLC (B89). Finally, Witte et al. optimized the eluent composition, flow rate, and temperature for the chiral separation of the racemic D-2 dopamine agonist N-0437 using response surface methodology and Smilde's multicriteria decision-making approach (B90).

Application/Compound-Oriented Reports. Ahuja discussed the optimization of selectivity, detectability, and analysis time from the perspective of pharmaceutical analysis (B31). Other reports included separations of (derivatized) amino acids (B91-B94) and selected metabolites (B95); peptides (B96); proteins (B97); hydroxylated triacylglycerols (B98); carboxylic acids in beverages (B99) and wine (B100-B102); organic acids (B103) and phthalates (B104); neurotransmitters (B105); steroids (B106, B107); phenolics (B108); flavonoids (B109); selenonium and arsonium cations (B110); sulfonamides (B111); and miscellaneous metabolites (B112-B114) and other compounds (B115).

C. DATA ANALYSIS

Undoubtedly one of the more valuable contributions to this section was Dyson's excellent monograph on chromatographic integrations methods (C1). Most if not all topics relevant to manual and electronic integration are covered thoroughly if not exhaustively in this well-illustrated work, including data acquisition, filtering and smoothing, detection/location of peak topographical parameters, Gaussian and exponentially modified Gaussian (EMG) models for chromatographic peaks, statistical moments, etc. An extensive discussion of the various sources of error for the commonly employed perpendicular drop and tangent skim algorithms for overlapped peaks is provided, including the largely unappreciated but significant effects of peak asymmetry and sloping baseline.

Acquisition-S/N Improvements. Ouchi reviewed the principles and methods of analog-to-digital conversion and data storage (C2). Reijenga discussed methods for data compression in isocratic LC (C3) and data acquisition and digital filtering in analytical isotachopheresis (C4). Felinger and co-workers described a method for signal-to-noise enhancement of chromatographic signals via digital smoothing with the Fourier transform (C5), including a new procedure for determining the correct value of the cutoff frequency. Results were compared with those of some earlier methods. Rice et al. employed a pump-probe differential thermal lens spectrometer to increase the signal-to-noise ratio in background-limited photothermal spectroscopy (C6); absorbances as low as $2 \times 10^{-7} \text{ cm}^{-1}$ could be detected. Finally, Duell and co-workers (C7) and Guiochon and Sepaniak (C8) exchanged comments on the minimum data acquisition rate and the benefits of stationary phase homogeneity for whole column chromatography.

Peak Modeling. The exponentially modified Gaussian (EMG) continues to be the model of choice in analytical-scale

chromatography, as evidenced by the number of citations in the comprehensive review (since 1983) of Jeansonne and Foley (C9). An important paper published subsequently by Berthod (C10) extends the EMG approach by allowing a variety of mathematical functions other than a Gaussian to be modified by a first order exponential decay function. In contrast, Olive and Grimalt (C11) reported empirical equations based on the log-normal distribution for the characterization of electronically measured chromatographic peaks.

Areas, Moments, and Deconvolution. Several important approaches to chromatographic peak characterization were reported during this review period. On the quantitative side, Synovec et al. (C12) described a precise, quantitative method of deconvolution based on the point-by-point ratio of sequential chromatograms that have been baseline corrected; neither peak modeling nor curve fitting is required. Cecil et al. (C13) compared factor analysis, Kalman filtering, rank annihilation, and other methods based on multiwavelength data for their quantitative accuracy and their ability to deconvolute overlapped peaks. Wu and co-authors used the EMG model and multivariate linear regression to develop equations based on the ratio of apparent peak heights and a novel asymmetry factor for the accurate quantitation of peak area ($\pm 5\%$) for severely overlapped peak pairs (C14) and also reported an improved approach for their deconvolution and calculation of statistical moments (C15). Maeder and Zuberbühler reported an improved algorithm for the nonlinear least squares curve fitting of multichannel absorption data (C16); their use of factor analysis prior to curve fitting resulted in significant savings in computer time and memory requirements. Yau and Kirkland presented an improved computer algorithm for the characterization of band broadening of skewed (but well-resolved) peaks (C17). Finally, Schure discussed important considerations in achieving "super-resolution" with the constrained iterative relaxation method (C18).

Peak Detection/Shape Analysis. A moderate increase in the awareness of the utility of peak shape was apparent during this review period. Cardot et al. reported a fully automated method for the detection and integration of chromatographic peaks (C19) and showed that detection limits based on the classical signal-to-noise ratio are less relevant for such systems. Arvidsson et al. (C20) developed a theory to explain the peak distortion they observed in the LC determination of omeprazole dissolved in borax buffer. Blo and co-workers employed the Edgeworth-Cramer series for the analysis of noise or peak shape in suppressed ion chromatography (C21) and field flow fractionation (C22) and developed useful rules for the latter to detect mixed elution processes, overloading, or polydispersity. Finally, Dose and co-authors showed how sorption isotherms could be determined from chromatographic peak shapes (C23).

Peak purity remained an important topic. Marr and co-workers developed a method of multiple absorbance ratio correlation (MARC) for assessing peak purity and identity in multiwavelength data sets that do not contain full spectra (C23a). MARC was less sensitive to the choice of wavelength than the conventional dual absorbance ratio; remarkably, its sensitivity was independent of chromatographic resolution. By treating the chromatogram as a curve in multidimensional space in which each point is the S/N ratio for one detector, Kalambet et al. (C24) were able to (i) analyze peaks for homogeneity, (ii) determine the number of substances in overlapped peaks, and (iii) determine individual elution profiles without prior knowledge of their spectra. Keller and Massart reported an improved method for the detection of impure peaks by using evolving factor analysis with a moving window and a fixed number of spectra (C25) instead of an increasing number of spectra as in the conventional approach. This adaptation was better in detecting low levels of impurities ($<1\%$). Karjalainen and Karjalainen (C26) reported a set of methods based on alternating regression that first solve for the concentrations and then the spectra of the sample components of a chromatogram. Applicable to both multichannel and single-channel data, the methods do not require libraries of retention times or known spectra. Finally, Bridge and co-workers developed and compared the reliability of a series of automated tests based on multiwavelength data for the detection of co-eluting peaks in isocratic RPLC (C27). The sensitivity of each test to the presence of a second component

varied with the resolution, relative size, and spectral similarity of the overlapping components.

Peak tracking, a requirement for many types of optimization schemes, was the subject of a few reports. Strasters and co-workers reported a strategy based on the multiwavelength detection and subsequent multivariate analysis of peak area and spectral data (C28). A combination of related factor analysis techniques was necessary to overcome insufficient resolution or lack of reference spectra and was used in the optimization of the separation of local anesthetics (C29). Lankmayr et al. (C30) summarized recent advances in the application of fuzzy theory for peak tracking in HPLC. This approach is based on the comparison of peak areas and elution order under widely varying chromatographic conditions. A simpler, but less robust approach of Molnar and co-workers (C31) that utilized only normalized band areas was adequate for the peak tracking of a ribosomal protein sample in gradient elution RPLC.

Factor Analysis. Only a few examples are noted here since an entire review is devoted to chemometrics elsewhere in this issue. Gemperline reviewed the capabilities and limitations of factor analysis to detect and resolve overlapped HPLC peaks using multiwavelength data from a diode array detector (C32). Shen et al. developed a rapid method for estimating the number of coeluting components in LC by visual inspection of the abstract factors or computer analysis of the powder density spectrum (C33). Whereas the chromatographic information appears as broad features in the abstract factor plot, it is present as a single peak in the low density region of the powder density spectrum. Msimanga and Sturrock utilized various parameters of factor analysis—autocorrelation coefficients, multiple determination coefficients, and single-vector uniqueness tests—as predictors of the number of major electroactive components in a matrix co-eluting peaks (C34). Pure component profiles were initially estimated by iterative target test factor analysis and then refined using least squares. Naish et al. (C35) utilized iterative target transformation factor analysis together with numerical differentiation to deconvolute and identify organic acids unresolved by HPLC. Spectral similarity was not a problem for resolution in excess of 0.5. A similar approach was used for quantitative analysis by Lee et al. (C36). Finally, Ohman and co-workers compared the success of partial least squares-residual bilinearization with rank annihilation factor analysis for the correction of background signals (C37).

Factor analysis can also be used to facilitate the interpretation of retention data. Wilce and co-workers utilized principal component analysis to study the retention behavior of 20 natural amino acids (C38). Walczak and co-workers employed correspondence factor analysis to interpret the retention process for 35 *E-s-cis* and *Z-s-cis* chalcone isomers on 20 normal-phase LC systems (C39). Righezza and Chretien performed a similar study with hierarchical ascending classification for 36 chalcones in 43 chromatographic systems (C40) and also discussed the limitations of principal component analysis and correspondence factor analysis (C41). Schmitz and co-authors characterized commercially available RPLC stationary phases using cluster, principal component (PCA), correspondence factor (CFA), and discriminant analysis and found PCA and CFA to be most useful (C42); phenol/aniline and toluene/ethylbenzene pairs were shown to be suitable for characterizing polar and hydrophobic interactions, respectively. Coenegracht and co-workers used PCA to examine the dimensionality and structure of 3 RPLC data sets and found that only two principal components (e.g., solvent strength and modifier selectivity) were needed to explain the total variance (C43).

Miscellaneous. McConnell et al. discussed a validation protocol for analog-to-digital interfaces (C44) and Cardone and co-workers (C45) discussed method validation from a chemometric perspective. Sreenivasan employed Shannon's equation to estimate negentropy and explain the retention of selected eluents in RPLC (C46).

D. NORMAL PHASE

Silica continues to be the material of choice for liquid-solid chromatography (LSC), although the retention behavior of anilines on alumina was described in one report (D1). Two reviews which focused on the chromatographic properties of

silica appeared (D2–D4). The latter (D3, D4), published in two parts, discussed in some detail the heterogeneous nature of silica surfaces and pointed out that, while silanols are certainly the active adsorption sites on silica, all silanols are not alike. Silicas will thus vary, even from batch to batch, depending on the relative abundances of different silanol groups. Wouters et al. (D5) described a simple and easy technique for measuring the surface area of silica that is available for chromatographic interactions. This simple technique is based on the adsorption of a methyl red dye, but is limited to silica gels with surface areas less than 300 m²/g due to the presence of micropores in gels of higher surface area.

Interest in fundamental retention processes on silica gel continues. Kowalska showed that the retention of 13 dihydroxylated aromatic compounds on an amino μ -Bondapak stationary phase using 2-propanol/hexane mobile phases could be predicted with a high degree of precision if intermolecular interactions among the components of the mixed mobile phase were accounted for (D6). Jaroniec continued to incorporate surface heterogeneity into retention models of LSC (D7). In this latest work, a continuous, two-dimensional energy distribution function is used to account for surface heterogeneity. If ideal solution behavior in the mobile phase is assumed, the model leads to a straightforward integral equation for the capacity factor. Netting and Rhodes studied the retention of long-chain lipids in silica columns using dichloromethane or acetonitrile plus half-water-saturated hexane mobile phases (D8). In these systems, the silica surface appeared to be covered by a layer of water into which significant amounts of polar modifier would absorb. The result was an immiscible stationary phase into which fatty acid pentafluorobenzyl esters and triacylglycerols partitioned. Correlations were observed between log (*k'*) and the number of double bonds in these solutes.

As in every biannual review of normal-phase LC, a number of studies appeared describing the role of the mobile phase in controlling retention. Borowko discussed the dependence of selectivity on mobile-phase selectivity and molecular size and shape (D9). Oscik-Mendyk studied molecular interactions in silica columns using a number of binary mobile phases (D10). Data was fit to a previously published model of Jaroniec, and the fitting parameters were used to verify the model. Borowko and Oscik-Mendyk also studied adsorption in ternary mobile phases (D11) and proposed a modification of the Jaroniec equation which takes into account adsorption of all solvents. Shatz and Kazoka (D12) presented data which suggested that both adsorption and partitioning was possible in silica columns. When the solubility of the polar modifier is high, characteristic adsorption retention behavior is observed. However, when the solubility of the modifier is low, a liquid stationary-phase layer apparently forms, leading to a mixed retention mechanism, changes in selectivity, and improved peak shape. Liquid-liquid partitioning on such dynamically-generated stationary phases was also the subject of a second article. Huber et al. (D13) showed that with proper selection of the solid support, little adsorption is observed, and when operated in this LLC mode, retention data was more similar than when the columns were operated in the LSC mode.

The addition of specific mobile-phase modifiers to achieve selective separations was the subject of numerous reports. Kowalska focused on alcoholic modifiers (D14) and showed that a regression of solute retardation vs mobile-phase composition yielded parameters which indicated the relative affinities of the solute for stationary and mobile phases. Lanin and Nikitin, on the other hand, focused on chlorinated hydrocarbon modifiers and their effects on the retention and selectivity of monoalkyl- and polymethyl-substituted aromatic hydrocarbons (D15). Kunugi and Tabei showed that reproducibility of retention times and peak shapes of 16 phenolic compounds were greatly enhanced through the addition of water to a hexane/ethyl acetate/acetic acid mobile phase (D16). Finally, crown ethers were used to enhance retention reproducibility and selectivity of chlorinated analogs of oxindole and isatin, commonly used starting materials in many pharmaceutical processes (D17).

Although a number of applications of polar bonded phases were noted during the period covered in this review, only a few fundamental studies appeared. Salotto and co-workers studied in detail diol-silica columns, and further compared

this bonded phase with amino- and cyanosilica (D18). In agreement with previous reports, they found the diol and amino phases essentially basic, while the cyano phase was mostly dipolar in character. The amino phase strongly retained acidic solutes. Unmodified silica and chemically-bonded diol, nitrile, and amino columns were tested for their ability to separate a series of ethoxylated nonylphenols using methanol or propanol modifiers in hexane (D19). The amino column gave the best separations, and regular retention behavior was observed with all modifiers. A group of 38 *E-s-cis* and *Z-s-cis* chalcones was used to probe the retention characteristics of nine polar bonded phases (D20). Amino, diol, and cyano phases had the greatest similarity to bare silica, while charge-transfer phases and a nitro phase offered unique selectivities.

Normal-phase enantiomeric separations using modified β -cyclodextrins were the subject of three reports. Armstrong and co-workers were able to resolve a number of enantiomeric pairs on β -cyclodextrin multiply derivatized with acetic anhydride, (*R*)- and (*S*)-1-(1-naphthyl)ethyl isocyanate, 2,6-dimethylphenyl isocyanate, and *p*-toluoyl chloride (D21). In a subsequent paper, these same workers described the enantiomeric retention behavior of (*R*)-(-), (*S*)-(+), and racemic [1-(1-naphthyl)ethyl]carbamate derivatives of β -cyclodextrins (D22). These workers concluded that enantioselectivity of these derivatized cyclodextrins was different than that of native cyclodextrin, where inclusion complexation is thought to play the dominant role. On the derivatized phases, two chiral recognition modes are operative. In some cases, the chiral selectivity of one configuration of the substituent and the cyclodextrin combine synergistically, while the chiral selectivity of the opposite configuration of the substituent and the cyclodextrin combine antagonistically. Pawlowska described a dynamical method for preparing permethylated β -cyclodextrins on silica from several alcoholic solutions (D23). The result is a chiral adsorption layer on microparticulate silica.

A number of normal-phase separations of polymers were reported. Styrene copolymers with methyl, ethyl, and butyl methacrylate and acrylate were separated on silica gel with a chloroform/ethanol gradient (D24). Poly(styrene-vinyl acetate) block copolymers were separated by chemical composition on silica gel with a 1,2-dichloroethane/ethanol mobile-phase gradient (D25). The copolymers were separated according to increasing vinyl acetate content.

Normal- and reversed-phase modes were compared for their ability to resolve a number of different compound groups. Resolution of polybutadiene isomers was better in the normal-phase mode using a cross-linked polyacrylamide gel and hexane/methylene chloride mobile phase than in the reversed phase mode with polystyrene gel and acetonitrile/methylene chloride mobile phase (D26). Other compound groups separated in both modes included styrene-methacrylate copolymers (D27), phosphonodipeptides (D28), and alkylanilines (D29). Kou et al. described a method for making a polymeric phase which could be modified for use in either normal or reversed phase modes (D30). (γ -Methacryloxypropyl)trimethoxysilane is the coupling agent, and modification with poly(1-vinyl-2-pyrrolidone) produced a phase which selectively retained aromatic nuclei.

An interesting application of normal phase LC was reported by Alimarin et al. (D31). These workers were able to separate Pt(II), Rh(II), Ir(IV), Ru(III), and Os(IV) as chelates with 8-hydroxyquinolone on a silica column with a methylene chloride/isopropyl alcohol mobile phase.

Two uses of the normal-phase mode to study physicochemical processes were noted. Normal-phase HPLC with propylaminosilica and mixtures of hexane and 2-propanol was chosen as a model for the interaction of benzodiazepines with their receptors in the central nervous system (D32). In a review of the works of A. V. Kiselev, Yashin pointed out that the electronic distribution in complex molecules can be predicted from normal-phase retention parameters (D33).

Finally, although instrumental developments are not normally a part of this review, two reports of detector methodology appeared which were of unique importance to the normal phase mode. Nonaqueous electrochemical detection of 13-*cis*-retinoic acid, *all-trans*-retinoic acid, acitretin, and vitamin A palmitate produced a detection limit of ca. 1 ng on column for these compounds (D34). Hexane, cyclohexane, and

dichloromethane mobile phases were studied in the thermospray LC/MS analyses of organophosphorous pesticides, chlorophenols, and chlorinated phenoxyherbicides (D35). By using positive and negative ion modes, detection limits in the low nanogram range were obtained. The sensitivity of the positive ionization mode was ca. 1 order of magnitude better than analogous sensitivity in reversed-phase LC.

E. REVERSED PHASE

Reversed-phase chromatography continues to be the most popular mode of analytical LC. This popularity in usage is reflected in the continued research interest in this mode of separation. Advances are being made in better understanding of the molecular mechanism of retention, in better synthetic schemes for reproducible preparation of the stationary phases, in the design of stationary phases with greater pH stability and less susceptibility to secondary retention processes such as those exhibited by basic amines, and in many, many new applications.

It is becoming more clear that the nature of the silica is one of the larger variables in the synthesis of traditional reversed-phase stationary phases. As well as pore diameter and volume, such criteria as type and concentration of trace metal impurities, and pretreatment procedures make a large difference, even when the exact same bonding chemistry is used. There were several reviews of the properties and characterization of small diameter porous silica during this review period. Berthod (E1), Berek and Novak (E2), and Nawrocki (E3, E4) all published excellent reviews of silica and its use as a reversed-phase substrate, with the two part series of Nawrocki being the most comprehensive (E3, E4). A special issue of the Journal of Chromatography also appeared which was solely concerned with liquid chromatography packings (E5); many of the papers in this issue will be referenced later in this section.

Two other general reviews appeared. Englehardt published on chromatographic characterization of surfaces and described the evaluation and differences among reversed-phase materials (E6). Staroverov and Fadeev published an excellent review of the preparation and structure of the bonded layer of reversed-phase materials (E7).

The remainder of this section is broadly divided into categories of stationary and mobile phases. This was done for convenience of organization only, and it should be stressed that chromatographic retention is a function of the thermodynamic difference in the chemical potentials between the two phases. The "solvophobic theory", which is still widely cited, does not account for the effects of the stationary phase. It bases retention on association of two solute molecules in a single solvent rather than on the transfer of a solute from one solvent to another. While important historically in that it provided the first physicochemical description of the retention process, it should not be viewed as a current level of understanding.

Stationary Phases. Bohmer et al. described retention by an extension of a self-consistent-field theory for adsorption to model surfaces with flexible grafted chains and the retention of chain molecules at these modified surfaces (E8). This theory is in disagreement with earlier statistical mechanical models of retention and is in opposition to some experimental evidence already in the literature. It will be interesting to follow attempts at validation of this theory.

There were three studies dealing with the effect of the length of the bonded alkyl chain. Hetem et al. published a two part study investigating the effect of chain length on stability of the resulting material (E9, E10). They experimentally confirmed that longer chains better protect the silica surface from attack by aggressive mobile phases and proposed a model for ligand and substrate hydrolysis. Atamna et al. investigated the effect of chain length and carbon loading on the separation of basic compounds (E11). Using a mobile phase of pH 2.0 they studied k' , R_s , α , and peak symmetry as a function of run time. Unfortunately, these columns were only characterized in terms of percent carbon, making the data only empirically useful. Many reports continue to appear in the literature using carbon content from CHN analysis as the descriptor of phase loading, and most commercial columns are described in this manner. It should be stressed, again, however, that this is *not* the relevant parameter and that these

reports are often uninterpretable. As Unger et al. (E12) pointed out over 15 years ago, carbon content alone is often misleading because of differences in the surface area of the original silica, which results in different surface densities of the bonded alkyl groups. Carbon content is only useful if reported with the surface area of the bare silica. Manufacturers and researchers are strongly urged to report phase loading as an amount per unit surface area ($\mu\text{moles of chains}/\text{m}^2$ of surface area).

Buszewski et al. investigated the effect of surface chain density on the retention mechanism and on the separation of some biological samples (E13, E14). Petrovic and Lomic conducted a similar study investigating retention of three homologous series and five nonhomologous solutes as a function of bonding density (E15). They also proposed a method for evaluation of the volume phase ratio, which continues to be a controversial issue.

The effect of the initial silica and pretreatment regimes on the synthetic process was also investigated. Hetem et al. prepared two stationary phases on the same substrate material, but pretreated one batch of silica with a HF acid solution (E16). They found the pretreated silica gave improved surface coverage and stability compared to those of the original substrate. Buszewski compared materials prepared on silica gel and on porous glass and characterized the materials by physicochemical and chromatographic methods (E17). Yamaguchi and Hanai prepared C_{18} , C_8 , and C_3 bonded silicas from ultra-high purity silica gel and compared the chemical selectivity of these phases to a polymer coated C_{18} silica and a C_{18} vinyl alcohol copolymer gel (E18). Akapo and Simpson also studied the effect of pretreatment of the silica substrate (E19). They prepared materials with and without silica deactivation, and characterized the resulting phases by ^{29}Si and ^{13}C cross polarization and magic angle spinning (CP/MAS) NMR.

Simpson et al. published a series of papers investigating the synthesis and properties of oligomeric C_3 materials (E20–E22). Stepwise silanization of porous silica with *n*-octylmethyldichlorosilane and subsequent hydrolysis of the unreacted chlorine atoms produced a dense-layered stationary phase. They found that at high loading values the capacity factor was independent of carbon load (E20), which is in agreement with previous work. Using a fluidized bed technique, they prepared phases consisting of from 1 to 10 oligomers, and found that those materials with ≥ 5 oligomers behave as a true dispersive phase and are far more stable to acid conditions than a typical reversed-phase material (E21). They further characterized the resulting phases by ^{29}Si and ^{13}C CP/MAS NMR (E22).

Kimata et al. developed a test scheme to evaluate hydrophobicity, steric selectivity and the extent of hydrogen bonding and electrostatic interactions from C_{18} materials and then prepared materials by different methods to give a data base for the evaluation of commercial stationary phases (E23). Okamoto et al. compared the separation of methotrexate in serum on C_{18} materials prepared from both porous glass and silica particles (E24). Seacrest noted an unusual property of a commercial column (E25). A Zorbax Rx C_8 column gave unexpected retention of nitrate, suggesting an anion-exchange mechanism. This observation raises an important question about the validity of the use of nitrate for the measurement of the void volume of reversed-phase columns.

There have been several reports of the use of spectroscopy for the characterization of reversed-phase materials. Men and Marshall reported the measurement of the interfacial polarity of a C_{18} material in methanol–water slurries by a fluorescence probe technique (E26). They reported that the regions sensed by the probes had an effective polarity comparable to that of 5–20% water in methanol and that there was no change in the effective surface polarity over the range of 2–50% water. Ellison and Marshall used ^2H and ^{14}N spin–lattice relaxation measurements to assess the surface fluidity of C_{18} surfaces (E27). They reported a surface viscosity of 4.2 cP, which is 12 times the value for neat acetonitrile. Buszewski used CP/MAS NMR, secondary ion MS, porosimetry, elemental analysis and chromatographic methods to compare porous glass and silica gel modified with mono- and difunctional octadecylchlorosilane (E28). In an especially exciting paper, Ilg et al. reported magnetic resonance imaging in a reversed-phase LC column (E29). They were able to observe

band profiles in a column completely noninvasively. The wall effect, which had been theoretically predicted, was confirmed for the first time, and thermal effects were directly revealed. Another especially useful paper was published by Sander et al., who used small angle neutron scattering to measure the thickness of alkyl-bonded silica surfaces (E30). They reported thicknesses ranging from 10 to 25 Å for various phases. Hetem et al. published an extensive study on artificial aging of mono-, di-, and trifunctional C_{18} materials on two different types of silica substrates (E31). Using solid-state NMR, elemental analysis and chromatographic techniques they showed that multifunctional materials had a higher resistance towards ligand stripping. The rigidity of the silica substrate greatly influenced the stability of the monofunctional phases.

Gilpin et al., published two papers dealing with characterization of reversed-phase materials using alkanolate and perfluoroalkanoate esters at test probes (E32, E33). They presented a thermodynamic description for the retention of homologous series and estimated the thermodynamic constant which described the sorption equilibrium of solvents between the mobile and stationary phases. For water–methanol, differences in composition between the mobile and surface phases were small, while for acetonitrile–water mobile phases there was a significant sorption excess of acetonitrile.

It has been well established that mono- and trifunctional derivatized silicas behave very differently, yet it is not always clear from commercial literature which procedure was used. Jinno presented a CP/MAS ^{13}C NMR method for the identification of the functionality of C_{18} stationary phases (E34). Miyabe and Orita described a method for the characterization of ligands by chemical cleavage with aqueous HF and analysis of the reaction products by GC, NMR, and MS (E35).

Because of the great variability in the retention and selectivity properties of the commercial reversed phase columns, there have been a plethora of methods proposed for their characterization. Engelhardt et al. reviewed previous test procedures, and found most were applicable only for the evaluation of the hydrophobic properties (E36). They said that the criteria for the description of “good” columns should be symmetric peaks for both neutral and basic solutes, and independence of retention on sample size. They proposed a test using a methanol–water mobile phase with phenol, aniline, and the three isomeric toluidines. Schmitz et al. performed a chemometric analysis of empirically selected test solutes for characterization of stationary phases (E37). They applied cluster, principal component, correspondence factor, and discriminant analysis to a data set consisting of k' , α , and asymmetry factors. Factor analysis confirmed the pragmatic finding that aniline and phenol are well suited for the characterization of polar interactions, and toluene and ethylbenzene, for hydrophobic properties.

Ying and Dorsey described a method for the characterization of the retentivity, or “strength” of reversed phase columns based on a value of $\ln k'_w$, the estimated retention of a solute in a mobile phase of 100% water, and the slope of the plot of $\ln k'$ vs $E_T(30)$, a solvatochromic solvent polarity measure (E38). They validated the method with 26 solutes varying in $\ln k'_w$ from about 2 to over 20, on 14 different reversed-phase columns. In agreement with previous work, they found the volume-phase ratio is the most important parameter in determining retention. Engelhardt and Jungheim developed a pragmatic test which differentiates between C_8 and C_{18} and which is also useful for studying column hydrolysis (E39). Kaibara et al. described a method for the measurement of hydrophobic retention characteristics based on the slope of plots of k' of selected aliphatic and aromatic solutes vs. the reciprocal of the methanol concentration of the mobile phase (E40).

Tanaka et al. presented an interesting paper showing microscopic characterization of commercially available packing materials (E41). They used tunneling electron microscopy to give information on the size, shape, and location of pores and the skeleton in a particle.

Miller and DiBussolo published a very interesting paper examining mobile-phase and stationary-phase parameters that influence pH stability of bonded stationary phases (E42). Shorter chain lengths, low bonding densities, or the lack of encapping reduced stability against base hydrolysis. Phases bonded to acid washed silica showed greater base stability than those bonded to nontreated silica. In opposition to previous

reports, the use of a silica precolumn did not serve to increase system usefulness, although the use of soluble silicates in the mobile phase did increase column lifetime for both short and long chain reversed phases.

The effect of residual silanol groups on the retention of basic solutes continues to be of interest. Hill compared two Zorbax columns, a traditional C_8 and a Zorbax Rx for efficiency and peak shapes for basic, neutral and acidic compounds (E43). While the Rx column performed better with traditional mobile phases, the addition of triethylamine to the mobile phase improved the performance of the traditional column to approximately that of the Rx. Welsch et al. synthesized a large number of butyl-, hexyl-, octyl-, and octadecylsilica stationary phases and investigated selectivity and peak asymmetry as a function of both the surface concentrations of silanol groups and the organic functional groups (E44). Matus and Ohmacht investigated the effect of endcapping on the separation of carotenoids on monomeric and polymeric C_{18} materials, and noted that for some separations the nonendcapped materials gave better separations (E45).

The synthesis of novel stationary-phase materials continues to generate significant interest. Buszewski et al. prepared a chemically bonded phase with a peptide group and compared the behavior to traditional C_{18} columns (E46). They noted the new column was especially useful for the separation of basic substances. Danielson et al. reviewed the use of both fluoropolymers and fluorocarbon-bonded phases and noted that the potential for the separation of biomacromolecules appears to be good (E47). Csato et al. prepared a pentafluorophenyl stationary phase and compared it to a traditional phenyl column (E48).

The use of multidentate ligands was investigated by several groups during this review period. Jinno et al. prepared a multidentate phenyl phase and investigated its utility for the separation of polyaromatic hydrocarbons (E49). Gilpin and Gangoda synthesized phases from 1,8-bis(dimethylchlorosilyl)octane and 1,8-bis(trichlorosilyl)octane and compared their properties to monochloro- and trichlorooctylsilanes (E50). Koziol and Grayeski prepared a tridentate ligand exchange stationary phase from trimethoxysilylpropyldiethylenetriamine and investigated its properties (E51).

Other unique phases which were reported include a poly(vinylbenzo-18-crown-6) immobilized silica (E52), a tertiary butyl alkyl phase (E53) and a dibutyltetramethyldisilazane phase (E54).

Tanaka et al. compared the selectivity of graphitic carbon packing materials to those of octadecylsilyl- and pyrenylethylsilica materials (E55). The carbon phase showed the highest hydrophobicity and selective retention of planar, unsaturated compounds.

The use of the word "polymeric" to describe stationary phases prepared from di- and trifunctional silanes, while historic, has become confusing. The introduction several years ago of polymeric resin based reversed-phase stationary phases has added a totally different meaning to polymeric stationary phases, and careful choice of descriptive words is necessary. Compared to the previous review period, there has been much more activity during this review period in the preparation and characterization of novel polymer based stationary phases. Two reviews of these materials appeared. Tanaka and Araki reviewed polymer-based packing materials (E56) and Schomburg reviewed polymer coating of surfaces for both LC and capillary electrophoresis (E57).

There were three reports of the encapsulation of silica gel by polymeric C_{18} materials. Hetem et al. (E58), Ohtsu et al. (E59), and Shirota et al. (E60) all prepared and evaluated these encapsulated C_{18} materials. In comparison to traditional silanized reversed-phase silicas, these materials are more stable and show fewer secondary retention effects.

Ohtani et al. prepared C_4 and C_8 bonded vinyl alcohol copolymer gels and compared them to previously prepared C_{18} gels and to traditional butyl-, octyl-, and octadecylsilane silicas (E61). Szabo et al. prepared and evaluated reversed-phase silicas from phenylmethylpolysiloxane (E62).

There were two reports dealing with the poly(styrene-divinylbenzene) resin phases. Lloyd gave a general overview of the properties of these materials (E63) and Pedigo and Bowers compared the retention behavior to alkyl-bonded silicas (E64).

Various other unique polymers were prepared and evaluated. These include copolymers from acrylates and vinylaromatics with different carbon chain lengths (E65), bis(methacryloyloxymethyl)naphthalene-divinylbenzene copolymers (E66), copolymerization of vinyl-modified silica with acrylic acid derivatives (E67), a toluene polymer (E68), and both poly(1-vinyl-2-pyrrolidone) and poly(1-vinylimidazole) bonded silicas with (γ -methacryloxy)trimethoxysilane as coupling agent (E69).

Restricted access packings have become popular only in the past few years. These materials are dual-mechanism stationary phases which have a hydrophilic exterior, with hydrophobic pores so that biological samples can be directly injected without protein precipitation. The unique feature of these packings is that they prevent the access of matrix components such as proteins while selectively retaining the drug components and their metabolites. Interestingly, in this review period there were more reviews of these materials than new primary reports! General reviews were written by Unger (E70), Pinkerton (E71), Perry (E72), and Feibush and Santasania (E73).

Haginaka et al. prepared a new internal-surface reversed-phase material having (*N*-octanoylamino)propyl groups bound to the internal surface of the porous silica and (*N*-(2,3-dihydroxypropyl)amino)propyl groups bound to the external surfaces (E74). They applied this new phase to the determination of nonsteroidal antiinflammatories and tricyclic antidepressants in blood serum. Kimata et al. developed a simple procedure for the preparation of these dual mechanism columns (E75). Partial decomposition of alkylsilylated silica with an aqueous acid was followed by the introduction of diol functionalities, and produced a phase which possesses greater hydrophobic character than other restricted access packings. Desilets et al. adsorbed polyoxyethylene hydrophobically through the use of nonionic surfactants and also covalently bonded it to reversed-phase LC packings, establishing a semipermeable hydrophilic layer over the alkylsilane surface (E76).

Also in contrast to the last review period, there was an increase in the interest in bonded-phase alumina columns. These materials may offer better pH stability than traditional silica particles, and much of the published work is directed toward understanding the retention mechanism of these materials. Haky et al. published a series of papers addressing these questions (E77-E81). These dealt with the assessment of mobile-phase flow resistance (E77), an evaluation of C_{18} bonded alumina for separations of proteins and peptides (E78), a comparison of polybutadiene-coated alumina and C_{18} silica for separations of proteins and peptides (E79), a comparison of C_{18} bonded silica and alumina for the separation of small molecules, and a comparison of C_{18} bonded alumina and other stationary phases for lipophilicity estimations (E81).

Arenas and Foley published an especially thorough evaluation of the retention mechanism on polybutadiene coated alumina columns, investigating the effects of mobile phase composition and temperature on retention and methylene group selectivity (E82). Pesek and Lin described an evaluation of synthetic procedures for the chemical modification of alumina (E83) and Park compared C_{18} bonded alumina and silica using linear solvation energy relationships and the Kamlet-Taft solvatochromic parameters (E84).

There is still a small interest in the use of dynamically modified silica to generate a reversed phase surface. Hansen has advocated this technique for years, and continues to be active, and published an excellent review of this technique (E85). Hansen and Tjørneulund also published a description of a new phase, generated by the adsorption of 3-(*N,N*-dimethylpalmitylammonio)propanesulfonate on silica (E86). Shatz et al. also described the adsorption of hydrazinium derivatives and the resulting chromatographies (E87).

Mobile Phases. In what may prove to be a highly useful study, Cole and Dorsey investigated reequilibration time following gradient elution reversed phase separations (E88). They found that the reequilibration time can be drastically reduced by adding 3% 1-propanol to both the strong and weak solvent. This provides a constant composition of propanol during the solvent gradient and a much more robust stationary phase solvation.

There were three careful studies of the thermodynamic driving forces for the partitioning process in reversed phase

separations. Sentell and Henderson investigated shape selectivity as a function of temperature and bonding density of C_{18} phases and found that subambient temperatures and high bonding density stationary phases gave improved shape selectivity (E89). They constructed van't Hoff plots ($\ln k'$ vs $1/T$) over the range of -20° to 70° and observed differences in the shape of these plots as a function of bonding density. Isaacs and Jaroniec also used van't Hoff plots to investigate the retention of homologous series of solutes on C_1 , C_4 , C_8 , and C_{18} alkyl chain bonded phases (E90). They noted that the enthalpy and entropy of transfer depended linearly on the number of carbon atoms in the alkyl chain of the homologous solute. A word of caution about these studies should be inserted here. Most temperature dependency studies are conducted over a very narrow temperature range of only about 30° . Linearity in van't Hoff plots can easily be found over this range, but it is becoming more clear that there are significant deviations from linearity if the temperature range is widened. Care should be taken to not draw too hasty conclusions based on limited temperature ranges.

Gheong and Carr used a set of infinite dilution activity coefficients for the alkylbenzenes in mixtures of water with 4 of the more common organic cosolvents to gain insight into the retention process (E91). Several important conclusions resulted from these studies. They showed that the stationary phase environment is considerably more polar than that of a bulk long chain alkane, which supports the idea that sorbed organic modifier plays a substantial role in establishing the chemistry of the stationary-phase environment. They noted that measurements of the activity coefficients of nonpolar solutes in methanol-saturated hexadecane are insignificantly different from those in pure hexadecane and concluded that the vastly different surface area to volume ratio of bonded and bulk phases is vitally important in bonded phase chromatography.

The use of solvatochromism as a probe of retention processes continues to generate interest. Michels and Dorsey used the $E_T(30)$ polarity scale for extrapolating plots of retention vs. organic modifier content to a hypothetical value of 100% water (k'_w) (E92). They recommended this process over the linear volume percent extrapolations that are commonly done. Zou et al. also investigated solvatochromic approaches to estimating k'_w but relied on the linear equation for extrapolation (E93).

Park et al. used the Kamlet-Taft solvatochromic approach to investigate retention processes (E94, E95). They measured hydrogen bond donor acidity for aqueous mixtures of methanol, ethanol, isopropanol, acetonitrile and THF (E94) and also studied the retention of monosubstituted phenols based on a linear solvation energy relationship (E95).

The use of polyaromatic hydrocarbons (PAH) as a probe for retention processes, especially as related to shape selectivity continues to be of great interest. These studies have been so successful that a Standard Reference Material (SRM 869) has been issued by the National Institute of Standards and Technology (NIST) for the evaluation of shape selectivity of reversed phase columns. Two reviews of these studies appeared. Sander and Wise published an excellent discussion of shape selectivity and approaches to assess this property (E96), and Jinno also reviewed the retention behavior of large PAH (E97). In new reports, Wise et al. noted some anomalous behavior for methyl-substituted PAH (E98), Li et al. discussed the retention of 10 PAH compounds using molecular weight, length-to-breadth ratio, intramolecular steric strain, and nonplanarity (E99), and Jinno et al. published two papers dealing with planarity recognition by C_{18} phases (E100) and studying retention behavior of PAH on a liquid crystal bonded phase (E101).

The determination of the void volume of reversed phase columns is a problem that still has not been satisfactorily resolved. Malik and Jinno presented a review of the methods which have been proposed and pointed out that the increasing use of cyclodextrin columns presents a whole different set of problems for this determination (E102). Alvarez-Zepeda and Martire derived equations which led to the evaluation and linkage of excess and absolute sorption isotherms of acetonitrile-water mixtures and then used these isotherms for the determination of the mobile-phase volume (E103). Vit et al. compared the values obtained by measuring the retention volume of phloroglucinol with the method based on the re-

tention data of a homologous series and that based on the use of isotopically labeled components of the mobile phase (E104). Montes et al. used free energy correlations between distribution coefficients and the number of carbon atoms found in a series of nine nitrosamines for determining the dead volume using both methanol and acetonitrile as the organic components of the mobile phase (E105).

The effect of the choice of injection solvent on chromatographic efficiency continues to generate interest. Hoffman and Chang reported that peak height and plate count were increased for some solutes dissolved in weak solvents and injected into strong mobile phases (E106). Vukmanic et al. (E107), Beaver (E108), and Zapata and Garrido (E109) all noted that the choice of injection solvent can affect peak integrity, including the production of split peaks and dramatic changes in chromatographic efficiency.

There have been many other approaches taken to studying retention and selectivity. Empirical modeling approaches for ternary mobile phases were published by Kowalska (E110-E112) and by Xie et al. (E113). The use of homologous series to probe selectivity continues to be used (E114-E117). Other methods of selectivity characterization include principal component analysis (E118) and a thermodynamic treatment using a displacement mechanism to model the sorption of solvents into the bonded phase (E119, E120).

Other methods used to study the retention process include three papers by Kowalska et al. on modeling of solute retention with methanol-water mobile phases (E121-E123) and two studies by Kaibara et al. on solute interactions with reversed-phase stationary phases (E124, E125). Individual studies were published on application of the UNIFAC method for assessment of retention (E126), the role of π - π interactions (E127), a calculation method for finding equivalent strength mobile phases with different binary solvent systems (E128), the use of solute and mobile phase partition coefficients to describe solute retention (E129), the use of bulk property parameters such as viscosity, dielectric constant, etc. in correlation analysis to describe retention (E130), and the use of information theory and "negentropy" to describe retention (E131).

Smith et al. continued work on the development of a retention index scale based on alkyl aryl ketones and the use of this scale to develop an expert system for the prediction of retention (E132-E137).

Finally, there were two papers dealing with the very practical question of choice of solute as internal standards in quantitative analysis. Yamauchi and Mori investigated about 70 phenolic compounds and recommended a series of 30 which showed a consistent order of elution regardless of the separation conditions (E138). Skelly et al. developed a computer assisted internal standard selection system with a database of 90 potential internal standards arranged in retention time order (E139).

F. BIOPOLYMER SEPARATIONS

High-performance liquid chromatography continues to have monumental impact on modern biopolymer chemistry as evidenced by the nearly 10000 citations of work published over the past 2 years that were retrieved in the literature search for this review. Thrusts were in the development of more efficient column packings, preparative HPLC, and applications in biotechnology and food science. Primary meetings that focus on new developments are the International Symposia on High Performance Chromatography of Proteins and Peptides and the International Symposia on Column Liquid Chromatography. Both alternate between the United States and Europe, and their proceedings, which are usually published in special journal volumes, are compendia of information on the topic (F1-F4).

Column Packings

Research continues to improve the stability and efficiency of column packings for biopolymer HPLC. The separation speed, efficiency, and operational stability of micropellicular 2- μ m silica-based stationary phases was demonstrated for biopolymer separations (F5). These studies included a molecular fur of octyl or stearyl chains for reversed-phase chromatography, as well as a hydrophilic layer with amino groups and polyethyleneglycol groups for ion-exchange and

hydrophobic interaction chromatography, respectively. Nonporous polybutadiene-coated silica microspheres were also prepared (F6). Polymer loads of 1–3 wt % which corresponds to approximately a 4-nm-thick layer, were found optimal, with no silanophilic interactions detected. Nonporous spherical resins were shown also to yield high protein recoveries when only nanograms of protein were separated (F7). A new type of hydrolytically stable reversed-phase material was prepared by multipoint covalent attachment of polystyrene to the surface of porous silica (F8). Mixtures of 10 proteins, some with molecular weights up to 240 000 Da were separated efficiently with aqueous TFA/acetonitrile mobile phases. A new group of ion exchangers was described in which ionic groups are exclusively located on linear polymer chains were grafted to the support surface. This tentacle-type arrangement of the ionic groups facilitates ionic interactions between the exchanger and biopolymer and minimizes nonionic interactions (F9). Mass transfer is thereby increased and distortion of the analyte peak decreased. Another new class of polymeric packings was introduced that allows 10–100 times higher mobile phase velocities than standard materials without loss in resolution. Chromatography with these packings is referred to as perfusion chromatography (F10). The increased mass transfer results from the pore structure which consists of 6000–8000-Å pores that transect the particle with a continuous network of smaller (500–1500-Å) pores. This geometry minimizes diffusion pathlength. Polyhydroxyethylaspartamide column packings for hydrophilic interaction chromatography of biopolymers were described and the retention mechanisms investigated (F11). In another study, hydrophilic interaction chromatographic separations of DNA were found to resemble the partitioning observed with aqueous two-phase systems based on polyethylene glycol and dextran solutions (F12). Nonporous, deformable agarose beads were reported to give efficient protein separations (F13), and there was no need to use small beads of narrow size distributions. When coated with insoluble metal compounds, such as ferric, aluminum, or zirconium hydroxides, these materials produced unique adsorbents for proteins, nucleotides and coenzymes (F14, F15). Because of alumina's wider range of pH stability, it continues to be explored as a matrix for biopolymer chromatography. A number of reactions were tested for the chemical modification of alumina. Only chlorination of the surface followed by reaction with organolithium compounds yielded sufficient coverage with organic material to be detected by Fourier infrared spectroscopy (F16). In another evaluation of octadecylalumina (F17), only 50% of the peak areas for cytochrome c were observed as compared to the areas found with octadecylsilica chromatography although resolution of octapeptides was superior on the modified alumina. The differences were attributed to pore size and shape of the alumina packing. Two-micrometer spherical particles of hydroxyapatite were applied to the chromatography of transfer-RNA and proteins (F18). Selectivities could be manipulated with sodium chloride and magnesium chloride buffers, but the latter destroyed the chromatographic properties of the packing. Selectivities similar to calcium hydroxyapatite were found with fluoride-modified zirconium oxide so that it too could be used as a biocompatible HPLC packing (F19). Stationary phases in which transition metals are chelated to an immobilized chelator were studied extensively to assess the effect of the structure of the chelator, the metal ion species, and the mobile-phase composition and pH on selectivity and retentivity (F20, F21). Unique separations may be obtained by careful manipulation of these parameters. Thiophilic adsorbents prepared from the reaction of mercaptoethanol and divinylsulfone-activated agarose were demonstrated to be especially useful for the single-step purification of immunoglobulins from crude serum although the mechanisms of selective adsorption were not understood fully (F22). Monolayers of amphiphilic lipids were covalently bound to silica particles were shown to be remarkably effective for protein chromatography, especially for the purification of membrane-bound proteins (F23). Polymeric macroporous membranes, 1 mm thick, gave resolution, loading capacity, and flow rates comparable to column chromatography, but with a hundred times less pressure (F24). High mass sensitivity (several nanograms of protein) was obtained by the use of short capillary columns and the optimization of gradient steepness (F25). Instrumental parameters were optimized to

produce a microprotein analyzer that gives real time analysis for monitoring the production of recombinant human growth hormone (F26).

Reversed-Phase Chromatography

Adsorption Phenomena. When a series of globular proteins was studied with gradient elution reversed-phase chromatography, the retention parameters were in the order: folded < surface-unfolded < urea-unfolded < reduced-unfolded protein structures (F27). These results agreed with literature reports that proteins with cleaved disulfide bridges undergo the greatest degrees of unfolding. Measurements of apparent first-order rate constants for refolding of ribonuclease A showed a greater correlation with pore size than with the type of reversed phase packing (F28). However, retention time correlated more with the type of surface modification. The changes of conformation of α -chymotrypsinogen were determined after incubation with reversed-phase packings with different alkyl chain lengths (F29). Enhancement of β -sheet structure was observed in all cases, but no differences were distinguished with alkyl chain length. The kinetics of adsorption of human serum albumin were studied by split-peak formation in mass-overload conditions. The adsorption rate constants with acetonitrile in the mobile phase were about one-fifth those in pure buffer, and the loading capacity decreased with increasing acetonitrile percentage (F30). Adsorption studies such as those described in this section provide a basis for optimizing conditions for efficient biopolymer separations.

Applications. It is impossible to cite but an infinitesimal sampling of the applications of HPLC in protein and nucleic acid chemistry. Selected examples are given to indicate the power of reversed-phase HPLC.

The simultaneous separation of the most common genetic variants of caseins and whey proteins in clarified skim milk were made in a single run with acetonitrile–water–trifluoroacetic acid as mobile phase. Quantitation was feasible for all of the milk proteins except the α_{s2} -casein variant (F31). A reversed-phase column in series with a size exclusion column and ion-exchange columns were used to assess the maturity of Gouda cheeses by measurement of changes in peak areas of caseins (F32). The cheese whey proteins from different animal species could be differentiated by with a mobile phase of water–acetonitrile–trifluoroacetic acid (F33) and a rapid method for determining the extent of whey protein thermal denaturation was described (F34).

Reversed-phase HPLC is now used commonly for the characterization of wheat proteins. It was used to identify rye gene translocation through reversed-phase chromatographic analysis of 63 wheat lines and 6 released cultivars (F35). Considerable attention is being paid to data analysis of chromatograms produced by these complex mixtures of proteins so that correlations with functional properties can be derived (F36–F38). With such chromatographic and discriminant analyses, techniques protein profiles of wheat samples were correlated with bread baking quality (F39), and the influence of such parameters as soil moisture (F40) and handling and processing conditions were assessed (F41). These methodologies are now being applied to the characterization of rice (F42) and oat proteins (F43). In another food application, reversed-phase HPLC of protein extracts were used to evaluate the quality of cod fish after various frozen storage treatments (F44).

To facilitate development of new applications of HPLC, novel instrument configurations are being developed. For example, a two-dimensional system with reversed-phase chromatography and capillary electrophoresis was devised that has a much higher resolving power and peak capacity than either of the systems when operated alone (F45). The system is automated and operated under computer control. Direct detection of proteins by electrochemical means was made much more expedient by on-line, postcolumn photolysis (F46). The inherent selectivity and sensitivity of electrochemical detectors may now be applicable to protein separations.

Hydrophobic Interaction Chromatography

In addition to the development of new column packings as described in the earlier section, effort continues to delineate

retention mechanisms of biopolymers in hydrophobic interaction chromatography (HIC). Preferential interactions of salts with column packings and proteins were proposed to explain the adsorption behavior in HIC and an equation derived which predicted capacity factor as a function of lyotropic number and salt molality (*F47, F48*). Binary and ternary salt gradients were found to modulate retention selectivity in HIC presumably through competitive salt-binding interactions with proteins and/or stationary phase (*F49*). For gradient elution HIC with the same salt, the initial salt concentration was found to effect resolution of proteins substantially (*F50*). A mathematical model of the elution process revealed that effect was due to the fact that retention time and axial dispersion behaved differently with initial salt concentration. In another investigation, water was demonstrated to be the displacing agent in the HIC process and an equation presented that related capacity factor of proteins to the water concentration in the mobile phase and the number of water molecules required to displace a protein from ligands (*F51*). In a comparative study of hydrophobic interaction, reversed-phase and cation-exchange chromatography of subtilisin variants, gradient elution HIC was demonstrated to be capable of separating proteins that differ by only one amino acid (*F52*). Reversed-phase was of much less utility for such separations. Selectivity in HIC was also mediated by the addition of surfactants to the mobile phase. Gradients with both salt and surfactant concentrations variable were preferable for repetitive analyses because re-equilibration times were shorter (*F53*). The applications of HIC for the separation and recovery of proteins and peptides are legion, so it is suggested that the reader search a bibliographic data base by protein name for specific applications.

Ion-Exchange Chromatography

Theories and models for protein adsorption onto ion exchangers continue to be proposed and refined. A new theory based on the solution of the linearized Poisson-Boltzman equation for two oppositely charged planar surfaces in contact with salt solution was tested with a large body of retention data. Chromatographically measured protein charges compared well with those obtained from titrimetric experiments (*F54*) although deviations were noted that resulted from non-uniform charge distribution. The adsorption of proteins by electrostatic interactions under conditions where the adsorbent had the same type of charge as the adsorbent was also reported. This was attributed to nonuniform charge distribution as well (*F55*). A model was developed which treated protein electrostatics in quantitative detail and took into account three dimensional shape and charge distribution (*F56*). When a comparison of protein retention times over a range of pH was made, a excellent correlation with the mean surface potential calculated from the model and retention time, while the correlation between retention time and net charge was much poorer. Wyman's theory of thermodynamic linkage was extended to ion-exchange chromatography. In one study, mixed electrostatic and hydrophobic interactions were characterized by ion and water release (*F57*). In another, the number of protein acid/base groups and exchanger ionic groups in the contact layer varied with mobile phase conditions, such that the number was higher when the interaction between protein and exchanger was stronger (*F58, F59*). In gradient elution ion-exchange chromatography of closely related protein variants, column residence time, and protein-salt interactions influenced chromatographic behavior (*F60*). A new approach, potential barrier chromatography, was described which exploits the fact that the depth of the adsorption well of interaction potential between adsorbate and adsorbent can become moderately deep when it controlled by opposing van der Waals attractive forces and repulsive double layer forces (*F61*). With appropriate selection of mobile phase pH and ionic strength, fast separations of protein mixtures were obtained with a two-step isocratic elution procedure. The addition of neutral water-soluble polymers was found to be particularly useful for separations of proteins with the same isoelectric points but different sizes (*F62*). Retention of proteins on ion-exchange columns was predictable and explained on the basis of the hydrophobicities of the polymer additives.

Ion-exchange HPLC is being increasingly applied to DNA and oligonucleotide separations. For example, it was shown that curved regions of DNA fragments were preferentially adsorbed to anion exchangers, suggesting a common dipole character to those regions due to the local accumulation of charges resulting from the compression in the minor groove of DNA (*F63*). The quantitative determination of drug-induced hypermethylation in human cancer cells was described and used to measure hypermethylation during chemotherapy (*F64*). Such applications may be extended with the use of computer-assisted retention prediction system for oligonucleotides which permitted selection of the proper gradient to maximize resolution and minimize analysis time (*F65*).

Silica-based anion exchangers for biopolymer separations can regenerate successfully with sodium hydroxide solutions containing aluminum ion. Wide pore polymer-coated packings were washed in excess of 100 cycles (*F66*), thereby increasing the utility of such packings for protein, DNA, and other biopolymer separations.

New system configurations were evaluated for improved biopolymer separations. A comprehensive two-dimensional system was presented that used a microbore cation-exchange column, operated under gradient conditions as the first column and size exclusion as the second (*F67*). The entire system was automated and under computer control. Peak capacity and resolution were improved over the individual columns and three-dimensional data representation provided a more reliable means of peak identification from run to run. (Diethyl-amino)ethyl groups were bound to the inner walls of hollow cellulose fibers and shown to have useful application to the fast flow anion chromatography of proteins (*F68*).

Interest in hydroxyapatite (HA) as a medium for protein separation continued during the present review period. The binding capacities of HA for globular proteins were found to depend on surface area of the adsorbent which, in turn, was influenced by method of preparation and conditioning of the adsorbent (*F69*). The number of HA binding sites that were covered by adsorbing molecule was weakly correlated with molecular mass (*F70*). It was concluded that the stereochemical structure of the adsorbate was discerned by the regular crystal structure of HA. It was demonstrated also that HA was composed of two types of binding surfaces and that acidic proteins adsorb on one type of surface and basic proteins on the other type (*F71*). Applications include the purification of antibodies using a ammonium hydrogencarbonate mobile phase (*F72*) and the separation of complex membrane protein mixtures as their sodium dodecylsulfate complexes (*F73, F74*).

Preparative Liquid Chromatography of Biopolymers

The separation and purification steps are crucial for the cost effective preparation of active proteins through bioengineering. Therefore there is substantial interest in preparative HPLC for this purpose. Since, in addition to resolution and time, sample throughput is a consideration in preparative chromatography, approaches for optimizing separations under mass-overload or nonlinear isotherm conditions were investigated by several researchers. The use of the Craig distribution model showed a simple relationship between sample size, bandwidth, and gradient conditions for both small molecules and proteins, but only 20-44% of the column capacity was available under separation conditions for macromolecules (*F75*). For proteins, separation in gradient elution as a function of sample size can vary appreciably with the nature of the sample, so that the relative dependence of retention on mobile phase composition in gradient elution reversed-phase chromatography must be determined for the sample of interest (*F76*). Similarly, studies of adsorption behavior of several proteins on an anion-exchange column demonstrated that the mobile phase had to be chosen carefully to optimize the combined effect of relative retention and of column saturation capacity on the production rate (*F77*). A new multivalent ion-exchange model of biopolymer chromatography under mass-overload conditions was developed and found to agree well with experimental observations (*F78*). Peak asymmetry was predicted and found to be lower with multivalent displacer ions than with monovalent ones. A rate equation model was presented that took into account axial dispersion, film mass transfer, intraparticle diffusion, and size exclusion effects, in addition to nonlinear adsorption isotherms

(F79). Mass-transfer effects were pronounced for systems with small sample pulse sizes and with long retention times. The model also allowed development of scaling rules.

Displacement chromatography was employed for preparative separation of a mixture of four proteins (F80). Under adsorption conditions where the isotherms crossed, the displacement system was unable to separate feed components. However, subtle manipulation of the adsorption conditions separated the isotherms enabling the purification and concentration of the proteins. In the sample displacement mode, the column is overloaded with sample until it is saturated with the protein of interest, while weakly binding impurities are displaced. By using two columns in series, crude preparations of soybean trypsin inhibitor were purified from both weakly and strongly binding impurities (F81). Successful scale up of displacement methods to larger particle and column diameters was achieved with no adverse effects on product recovery (F82), thereby increasing further the economic advantages associated with displacement chromatography. The most efficient means to recover pure β -galactosidase and optimize recovery was to use displacement followed by frontal chromatography (F83). Conventional elution ion-exchange chromatography was compared with displacement chromatography for the isolation of lactate dehydrogenase from a mixture. In general, displacement gave better results in terms of fraction purity and specific activities (F84). New methods were presented for determining competitive adsorption isotherms using multicomponent frontal experiments and compared to previous ones for their utility in modeling preparative separations (F85).

The new, very wide pore polymeric packings that lend themselves to fast flow separations offer advantages for preparative chromatography (F86) because the improvement in intraparticle mass transfer allows higher flows without substantial resolution loss. The continuous separation of proteins was possible using an annular column configuration (F87). The separation performance was essentially the same as that of a conventional chromatography. The use of modules containing from 120–27 000 hollow fibers were applied to preparative separations of proteins with reversed micelles as stationary phase (F88). These novel systems could prove to be important since they can be operated also under overload conditions (F89).

G. AFFINITY CHROMATOGRAPHY

Reviews. Applications of affinity techniques for the isolation of biomolecules continue to expand. Overviews of general methods (G1) and specialized techniques were published (G2). The performance characteristics and properties of support matrices for improved affinity separations were explored (G3). The theoretical aspects of high performance affinity chromatography for both the isolation and the analysis of biomolecules were highlighted in a book chapter (G4) and the engineering aspects of affinity preparative separations were reviewed (G5). A comparison of methods for measuring antibody affinity was reported (G6).

Bioadsorption. A mathematical model describing biospecific adsorption processes was numerically solved and found to compare favorably with published experimental data and with theoretical predictions (G7). A systematic study of nonlinear effects on the determination of the affinity binding constant produced a universal function for zonal analysis that was found to be strongly correlated with experiment (G8). A combination of experimental studies and numerical simulations of frontal chromatograms led to a simpler method for characterizing solute-matrix interaction kinetics in affinity chromatography (G9). When small (12- μ m) silica beads were used as a matrix for immobilization of dye, rapid adsorption rates for proteins were observed but the adsorption of different proteins could be described by different type isotherms (G10). Binding studies coupled with circular dichroism data showed that the binding of peptides to anti-peptide antibodies was dominated by the number and pK_a values of the charged residues in the peptides (G11). Thermodynamic and kinetic investigations on rigid and soft affinity gels with varying particle and pore sizes demonstrated that the use of small size adsorbents for affinity preparative isolation of proteins may not be justified because identical breakthrough profiles were

observed with both large and small sized adsorbents (G12). Nonporous affinity supports displayed nearly rectangular breakthrough shapes at the onset of adsorption, but slow adsorption kinetics became evident as saturation was approached. This was attributed to surface rearrangement of adsorbed proteins especially on adsorbents of high ligand density. Deformed nonporous agarose beads were used for immuno-affinity purification of antibodies against human growth hormone. Desorption with 60% ethylene glycol containing 1 M NaCl gave total recovery of the antibodies (G13). It was suggested that cooperative hydrophobic and electrostatic interactions may have been involved in the antibody-antigen binding.

Immobilization. The chemistry for the attachment of ligands for the bioadsorption of analyte continues to receive the attention of researchers. Using hydrazide chemistry for the site-specific coupling of antibodies both to agarose and to silica, it was demonstrated that although covalent bonding was responsible for the stability of the linkage of antibody to surface, physical adsorption forces dominated the orientation of the antibody (G14). These forces were manipulated to alter the properties of the bioadsorbent. Modification of the dimethyl pimelimidate cross-linking method led to protein-A based immunoaffinity columns that did not leak IgG at low pH and that were capable of binding antigen at 80% of their theoretical capacity as compared to the 20% binding capacities reported previously (G15). New linkers based on diamino-hydroxyhexane and cystamine chemistries were described that were stable under all conditions employed in biochemical research. The former could be cleaved however with periodate and the latter with dithiothreitol so titers and binding processes could be evaluated (G16). It was demonstrated also that antibodies could be immobilized on octadecylsilica by incubation at 12 °C in phosphate buffered saline. After free binding sites were inactivated with serum albumin, the immobilized antibody could be dissociated only with surfactant solutions (G17). A novel conducting polymer was deposited on silica and evaluated as a stationary phase for affinity chromatography. The potential applied during the binding and elution stages influenced recovery and purity (G18). Biotinylated proteins and antibodies were attached to avidin-Sepharose and shown to give better capacities and product yields than those achieved with conventional chromatography on CNBr-activated support (G19). A newly activated support, a tresylpolymer, was evaluated for coupling of antibodies. Antibody coupling yields exceeded 80% and antigen binding efficiencies of 70–80% were achieved, probably because of a selective attachment of the Fc region of the antibodies (G20). The type and concentration of coupling buffer was found to be important in the coupling of protein to tresyl-activated supports. In general phosphate buffer at 1 M concentrations and higher was most effective (G21). The advantages of traditional polysaccharide-based supports were combined with the excellent mechanical properties of silica by coating the porous silica beads with a double layer of polysaccharide (G22). Acidic proteins were not adsorbed to the double layered silica, which could be activated by classical methods and coupled with active ligands. The physical properties of the matrix were important in preparative affinity chromatography (G23). The studies showed that binding capacity increases with increasing pore size and then decreases as the pore size increases to become sufficiently large to significantly decrease surface area. The final bonded phase pore diameter of at least 200 Å is desirable. A silica-based reactive aldehyde matrix minimized nonspecific protein binding while preserving high binding capacity. A Schiff base reaction involved in the coupling of a ligand to the affinity medium was rapid, allowed the use of mild conditions during coupling and resulted in a stable linkage (G24). Functionalized and reactive radioderivatized polystyrene can be obtained by exposing the polymer to 10–100-kGy radiation. The surface-immobilized ligands that were obtained by radioderivatization were capable of binding antibodies (G25). The use of charged spacer arms interdispersed with ligands has been proposed as a means of easing the dissociation problem. While somewhat successful, this approach complicates ligand coupling reactions and could lead to enhanced binding if the charge and its arm length are not optimal for the separation required. These disadvantages set clear limits on the utility of affinity-repulsion chromatography (G26).

Using an immunoaffinity model with IgG as ligand and goat anti-human antibodies, the efficacies of 13 elution reagents were compared. The best eluent for this system was 3 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.075 M HEPES/NaOH with 25% ethylene glycol, pH 7.2 (G27). Also, the specific activities of antibodies recovered from experiments performed under antibody excess and antigen limiting conditions were 3–8 times higher than those produced by antigen excess and antibody limiting configurations. A sulfobetaine surfactant also was found to be an effective desorber in affinity chromatography (G28).

Applications. Selected applications of the many found in the literature are presented to illustrate concepts and the increasing role of affinity techniques in analytical chemistry and in preparative biochemistry.

Glycoproteins known to contain O-linked oligosaccharides were found to bind to columns of the immobilized, lectin, jacalin. Binding of proteins did not require divalent cations and was affected little by changes in pH or ionic strength (G29). The types and chain lengths of the spacers were important variables in the affinity chromatography of carbohydrate binding proteins (G30) and lectins (G31). For the latter, the association constant increased with number of saccharide units implying that not only the nonreducing monosaccharide residue but also the interior portion of the sugar chain took part in complexation. Wheat germ lectin immobilized on a diol-bonded silica column gave improved resolution of human bone from liver alkaline phosphatase isoenzyme (G32). Results with chromatography correlated significantly with those with solid-phase immunoassay using monoclonal antibodies and therefore provided a method to expedite monitoring of patients with cirrhosis and diverse metabolic bone diseases.

Monoclonal antibodies in complex mixtures were quantified with high performance affinity chromatography utilizing protein G as ligand. The results agreed to within 10% of a standard enzyme-linked immunospecific assay, although the chromatographic method required only minutes instead of the hours for the ELISA (G33). Antibodies to human growth hormone were determined by protein G affinity columns used in tandem with reversed-phase columns and fluorescence detection. The recovery was 98%, and the standard deviation for the analysis of serum samples was 1–8% (G34).

Fast detection of attomole amounts of human growth hormone was accomplished by the use of columns which were sequentially filled with two different immunoaffinity resins. This sensitivity was 100–1000 times better than classical methods (G35). High-performance chromatography with antibodies that specifically bind glucose-containing oligosaccharides permitted the analysis of a glucose tetrasaccharide in serum and urine in less than 20 min without derivatization using pulsed-amperometric detection. The method represented substantial improvement over previous methods (G36). The mycotoxin, zearalenone, could be measured in milk using an immobilized monoclonal antibody column for sample clean up and ELISA detection. Mean recoveries were 95% and interwell and interassay relative standard deviations were 14.5 and 9.1%, respectively (G37).

Affinity chromatography was used not only for analysis and isolation but to characterize interactions of biomolecules. Equations were derived which describe the partitioning of acceptor between matrix-bound and soluble forms in terms of total, rather than free ligand concentrations. In addition to simplifying the performance of binding experiments, the development makes possible the application of affinity chromatography binding studies to systems characterized by high binding affinities (G38). Results for the binding of heparin to antithrombin III were in good agreement with those obtained by fluorescence quenching measurements. The interaction of calcium with casein submicelle was investigated with frontal chromatography on columns of submicelles immobilized on succinopropyl controlled pore glass (G39). The results suggested that calcium salts bound to the submicelles with greater affinity than free-calcium. Columns of immobilized *trans*-retinal differentiated native and denatured β -lactoglobulin in mixtures of milk proteins and in whey (G40). The amounts of native β -lactoglobulin determined by affinity chromatography were in agreement with values found by solubility measurements.

Dye-binding affinity chromatography continued to receive the attention of researchers. Triazine dyes were used for the single step purification of lactate dehydrogenase from heart

muscle extract. As expected the length of the spacer influenced binding capacity and recovery (G41). Purification (99%) of a penicillin-binding protein of *Escherichia coli* was accomplished in the presence of β -lactams with triazine dye columns (G42). Selection of the proper dye for selective binding requires considerable experimentation. A method was described that allows the simultaneous testing of 96 different dye adsorbents for their binding ability and to test their biospecific elution (G43). Small amounts of cell-free extract were applied to the adsorbents which were packed in a 96-well transplate cartridge. After elution, the amount of eluted enzyme is tested in a microtiter plate assay. Computer-aided molecular design was exploited for design of dye molecules which show predictable selectivity (G44). Novel perfluorocarbon emulsions and microparticulate supports with adsorbed dyes offered new potential for enzyme purification (G45). Specially designed biomimetic dyes bound to monosized support materials were found highly efficient isolations of intestinal alkaline phosphatases and urine urokinases from crude extracts (G46).

Immobilized metal ion affinity chromatography systems were used in the displacement mode for the simultaneous concentration and purification of proteins (G47). This work demonstrated that other proteins could be used as displacers under appropriate conditions and that the displacement behavior is due to the specific interactions with the metal. An innovative approach was described that was based on the hypothesis that a specific chelating peptide (CP) with a high affinity for an immobilized metal can be used to purify a recombinant protein by extending its gene sequence to code for the extra amino acids in the CP (G48). When CP is attached through an enzymic cleavage site it could be removed after the chromatographic purification step. The solute structure, ligand density, and mobile-phase salt concentration were shown to influence the retention of synthetic hormones on Cu(II) high-performance affinity columns prepared from two different commercial chelating matrices. At 2–4 M NaCl concentrations aromatic interactions were superimposed on the metal affinity characteristics of the hormones and the results suggested that the presence of aromatic and hydroxy amino acids further increased the strong binding of hormones containing histidine, tryptophan or cysteine (G49).

By using F-actin affinity columns to select proteins solely by their ability to bind to actin filaments, over forty proteins were identified and purified from early *Drosophila* embryos (G50). This technique permitted the study of actin structure generation in the developing cells. Transcription-promoting proteins were isolated by circulating a partially purified fraction between two coupled columns, one containing random sequences of prokaryotic DNA and the other a specific cloned fragment. This method should find general application in the purification of factors that regulate biological processes (G51) by binding to specific sequences of DNA. Mechanism-based affinity chromatography is based on the adsorption of the desired enzyme to an immobilized inhibitor which functions in the presence of substrate in the mobile phase (G52). The utility of this approach was demonstrated by the purification of a herpes simplex DNA polymerase. Desorption is affected by removal of the substrate from the mobile phase.

Immobilized anti-peptide antibodies that were prepared against the synthetic tetradecapeptide having the same sequence as the putative binding site of the rabies virus was able to recognize the whole virus and its peplomeric glycoprotein (G53). These affinity columns gave selective and efficient purification of the virus glycoprotein without denaturation. Discrepancies in binding behavior of monoclonal antibodies raised against synthetic peptides based on sequences of several proteins in solution and immobilized were observed. As a consequence binding of such antibodies to protein does not infer that the peptide has assumed a conformation that corresponds to a cognate sequence in the native protein (G54). In affinity chromatography, the pH and ionic strength dependence of binding to synthetic peptides was dominated by the number and pK_a values of the charged residues in the peptides (G55).

For preparative-scale enzyme purifications, process time was reduced by carrying out the bioadsorption from a large quantity of dilute solution in the batch mode. Purification and elution were continued with columns of bound enzyme (G56). Centrifugal affinity chromatography resulted in a 3-fold

reduction in the time required for the purification of human IgG (G57). A continuous protein purification process with affinity recycle extraction was described and mathematical models developed to assess its performance (G58). Affinity membranes mounted in a radial flow cartridge allowed high throughput of human plasma (20 mL/min) with 100-fold concentration of plasminogen and 85% recovery (G59).

H. ION CHROMATOGRAPHY

Ion chromatography (IC) continues to generate much interest, both in research directed toward improving the technology and especially into new applications. Except for those reports devoted exclusively to the development of one or the other of the methodologies, we will not distinguish between suppressed and nonsuppressed techniques. During the period of this review, there have been advances made in the design and synthesis of ion-exchange stationary phases, in the understanding of the retention process, in the development of better mobile phases for certain applications, and in new detection techniques. Compared to the last review period, there has been relatively little work published on suppressor technology.

Several excellent reviews of this technique appeared during this review period. Small, who is credited with the invention of suppression technology for IC, published a historical account of the development of ion chromatography (H1). Pietrzyk published an exhaustive and excellent review of ion exchanger types and their uses (H2), and Fritz published a review of the principles and applications of ion-exclusion chromatography (H3). Two reviews of inorganic ion exchangers appeared (H4, H5), and Dasgupta published a critical review of postcolumn manipulation, including suppressor design, replacement ion techniques, indirect detection and postcolumn reactions (H6). Two reviews of detection in IC were published, one a general review by Rocklin (H7) and the other by Jandik et al. specifically dealing with electrochemical detectors (H8). There were also reviews of environmental applications of IC (H9), of sample cleanup methods (H10), and of the use of macrocyclic ligands in IC (H11).

The use of gradient elution in IC is generally difficult either because of exhaustion of suppressor capacity or the effect on the baseline in nonsuppressed IC. Jandik and Gjerde et al. published a series of articles describing the use of a postcolumn solid-phase reagent which is a suspension of an ion-exchange resin of the opposite charge to the analytical column (H12–H14). Dasgupta's group described a membrane-based electrochemical approach for the *in situ* production of ultrapure ionic substances (H15). This allows gradient generation without mechanical proportioning.

The study of isotherms also received attention during this review period. Lucy et al. discussed peak interactions under concave isotherm conditions in preparative IC (H16). Two types of peak interaction have been identified, the retainment effect, where a more retained peak shifts to greater retention times due to the overloading of an earlier peak, and the pull-back effect, where a peak is smeared into a more retained peak due to the overloading of the later peak. Takahashi and Goto measured adsorption isotherms of three amino acids by a batch technique and then measured kinetic parameters by a moment method (H17). Tsuji and Komarneni described a new method for the evaluation of the distribution coefficient (K_d) at infinite dilution, based on extrapolation of a Kielland plot from an ion-exchange isotherm to zero loading (H18).

There was also interest in theoretical treatment of peak shapes and system peaks. Blo et al. used numerical analysis to study the performance of a suppressed IC system (H19). Peak-shape analysis was used to establish the linear calibration range, and noise evaluation was performed to establish the detection limit. There were two studies of analyte and system peaks in IC. Sato studied this phenomenon for bulk property detectors (H20) and used both computer simulations and experimental investigations to verify the acid partition model of Jackson and Haddad. Yamamoto et al. investigated the appearance of sample and system peaks for photometric detection and derived an equation expressing the sample response-capacity factor relationship at low mobile phase pH (H21).

The understanding and prediction of the retention process continues to be of interest. Haddad and Foley (H22) derived

a retention model for inorganic cations with eluents containing a single competing cation and a complexing ligand. Good agreement was found between predictions and experiments for a low-capacity fixed-site cation exchange column, but the agreement was not as good for ion-interaction chromatography on a C_{18} column. Sasagawa et al. presented a method for predicting isocratic capacity factors from two initial gradient runs (H23). They did not assume linear solvent strength (LSS) conditions, which can cause significant error in k' predictions in ion-exchange chromatography. Watanabe and Kubota studied electrostatic behavior in connection with anion exchange reactions by measuring streaming potentials in a flowing system (H24). They showed that the electrostatic behavior influences the selectivity coefficient of the resin. Daignault et al. studied the relationship between retention of anions and their polarizability (H25). Rahman and Hoffman studied the retention of organic cations and found that plots of k' vs the reciprocal of the exchangeable alkali metal ion concentration were linear (H26). They interpreted the slopes and y intercepts as indicating two sites of solvophobic interaction occurring in addition to the electrostatic interaction.

Shan et al. proposed a new method for the measurement of limiting ionic equivalent conductance by single column IC which involves preparing a calibration graph with ions of known conductance at different pH values (H27).

Mobile Phases. Johnson reported an interesting (and frustrating!) set of unexpected behavior for a separation of sulfite and sulfate with a bicarbonate mobile phase (H28). He described on column equilibria which, as the sulfite concentration increased, favored formation of disulfite and carbonates. Other mobile phases did not exhibit this problem.

The simultaneous separation of anions and cations continues to be of interest. Saari-Nordhaus and Anderson developed a method using an anion and cation column and a switching valve (H29). Three eluents were developed to allow simultaneous analysis of anions and monovalent cations of anions and divalent cations. Tarter discussed the choice of eluent for these simultaneous separations and showed examples of problems that can occur from eluent-analyte interactions, pH of the mobile phase, and eluent peaks which may result (H30). Yan and Schwedt showed the use of chelating agents EDTA, DCTA, and others which allowed the simultaneous separation of inorganic and organic anions as well as alkaline earth metal cations (H31).

Similarly, the separation of monovalent and divalent cations has proven problematic. Rocklin et al. discussed three methods by which this can be accomplished (H32). These include gradient elution, eluent step changing, and column switching. Yan and Schwedt described a simultaneous separation of alkali, alkaline earth, and heavy metal ions on a weak cation exchange column with a mobile-phase combination of tartaric and oxalic acid (H33).

Marheni et al. described a matrix matching approach for dealing with samples containing a high level of a matrix ion (H34). They showed the separation of 10 UV absorbing anions using a NaCl mobile phase containing 5 mM phosphate buffer and showed that the performance of the separation was virtually unaltered for sample chloride concentrations in the range of 0–20 000 ppm! A similar approach was applied to samples containing elevated levels of sulfate.

The choice of mobile phase ion also continues to generate much interest. Vautour et al. evaluated the relative efficiencies of substituted aromatic monocarboxylic acids as eluents for the separation of inorganic and organic anions (H35). Hirayama et al. described the use of β -diketonate anions for the elution of weakly retained anions (H36). Because of the chelating ability of these species, the influence of metal cations in the sample solution was eliminated. Singh et al. developed a new mobile phase for the separation of Mg(II), Sr(II), and Ca(II) in high salinity subsurface waters (H37). Thomsen and Cox published a very interesting paper evaluating a homologous series of alkanesulfonates as eluents in the IC separation of nitrate and nitrite (H38). The alkyl chain length has a large effect on eluent strength with poly(styrene-divinylbenzene) based stationary phases, but this effect is much less with silica based columns. Sulfonates from methane- to 1-hexanesulfonate gave excellent separations of nitrate and nitrite, even when one was present in 1000-fold excess over the other. These mobile phase ions are also compatible with conductivity

detection. Danielson et al. described the use of vanadyl and vanadate salts as useful mobile phases for cation and anion separations using indirect detection at 254 nm (H39).

Smith et al. evaluated the use of temperature programming in macrocycle-based IC (H40). Because the complexation of metal ions with macrocycles is exothermic, retention is decreased with increasing column temperature. Temperature programming improved separations compared to isothermal conditions.

Stationary Phases. The development of new stationary phases and the characterization of existing phases generated much interest during this review period. A highly novel anion-exchange material was described by Deinhammer et al. (H41). It consists of polypyrrole-coated glassy carbon particles and is connected as the working electrode in a three electrode electrochemical cell arrangement. Electrochemically induced changes between the neutral (reduced) and cationic (oxidized) forms of polypyrrole lead to the transformation from a neutrally charged stationary phase to one with a high positive charge density. They report that the potential can be varied to develop separations analogous to gradient elution, except here they are using a programmable stationary phase. Developments of this material will bear watching.

Jenke evaluated the efficiency of six commercially available IC columns and found optimum efficiency in the flow rate range of 0.3–0.7 mL/min, following classical Knox type behavior (H42). Walker et al. compared silica-based and polymer-based strong ion exchangers for the separation of organic ions using indirect UV detection (H43). The two materials gave significantly different retention properties, with the polymer based material providing both adsorption sites and ion exchange sites, while the silica based material showed predominantly ion exchange interactions. Pietrzyk et al. studied anion and cation separations on mixed-bed columns of both polymer based and silica based materials as well as materials that contain both anion and cation groups (H44). Oberdorfer et al. studied the interaction of weakly acidic monosaccharides with a polystyrene sulfonate in the H⁺ form and found unexpected selectivity for epimeric aldohexoses, deoxyaldohexoses, and deoxyfluoroaldohexoses (H45).

The use of silica and alumina as a substrate for IC columns continues to be of great interest. Lin et al. described the immobilization of a quaternary ammonium salt of cyanuric chloride onto the surface of silane modified silica, giving a low-capacity anion-exchange column (H46). Bonn et al. described the covalent bonding of iminodiacetic acid to porous silica and investigated this material for the separation of transition metals (H47). Tong et al. prepared an aryl sulfonic acid derivatized silica and applied it to the separation of some organic and inorganic ions, including the lanthanides (H48). Secreast described the unexpected retention of nitrate on a Zorbax Rx C₈ column and found significant retention and separation of anions (H49). This column was used to separate the amine plus counter anion components of some research pharmaceutical salts by a mixed mode reversed-phase, anion-exchange mechanism. Buchberger and Winsauer described a novel coupled column approach using alumina to concentrate sulfate or iodide from complex matrices and minimizing sample cleanup (H50). They reported that the selectivity of alumina was significantly different than traditional quaternary amine type materials, making it well suited for online column coupling techniques. Shirai et al. described the photocrosslinking of poly(cinnamoyl crown ether) onto silica gel, and the use of this material for the separation of both alkali metal cations and anions of potassium salts (H51).

The preparation of novel polymer based materials also continues to be of interest. Saari-Nordhaus et al. evaluated a hydroxyethyl methacrylate-based macroporous copolymer with quaternary amine functional groups for the separation of anions by either single column IC or suppressor-based IC (H52). Smith and MacQuarrie evaluated the properties of a latex bonded pellicular anion exchanger (H53). These materials contain ion exchange sites in a pellicular form combined with adsorption sites on a neutral polymeric core bead. Retention can be controlled both by the ionic strength of the eluent and also by the amount of organic solvent. Liu et al. described the preparation and properties of a cation exchanger made by anchoring *N*-(hydroxymethyl)thioamide on acrylonitrile-divinylbenzene copolymer (H54). They measured the sorption properties for mono-, di-, and trivalent metal ions

and evaluated the suitability of the material for metal preconcentration as well as analytical scale chromatography. Jonas et al. described the preparation of a chelating resin composed of 8-hydroxyquinoline immobilized on a poly(styrene-divinylbenzene) copolymer matrix (H55).

Dynamically coating other materials to create ion exchangers continues to be of interest. These methods are useful when the need for an ion exchange separation is sudden and infrequent. While these dynamic methods are attractive, great care must be exercised with temperature control and other chromatographic conditions, as this will affect the amount of adsorption of the active material on the support and then the day-to-day reproducibility. Ito et al. developed low capacity anion exchange columns by sorption of cetyltrimethylammonium on two types of C₁₈ columns and applied them to the separation of inorganic iodide species (H56). Walker studied the separation of inorganic analyte anions by columns prepared by coating a dye onto both a polymer based packing and onto a C₁₈ column (H57). Mueller and Meisch coated C₁₈ columns with methylene blue, methyl green and crystal violet to obtain anion exchange columns with adsorbed quaternary ammonium groups (H58). The crystal violet gave a "permanently" coated column which allowed efficient separation of anions without the addition of dye or organic modified to the mobile phase. Jones and Schwedt described the formation of permanent coatings of triphenylmethane type dyes on neutral polystyrene resins (H59). Bromophenol blue gave a coating with separation properties similar to those of a low capacity sulfonated resin, although with lower efficiency. Chrome Azurol S produced a chelating exchange coating which was studied for both di- and trivalent metal species. Lamb and Drake coated hydrophobic macrocycles onto C₁₈ derivatized silica or polystyrene columns and then used an aqueous eluent containing cations which bound dynamically to the macrocycle, forming positively charged exchange sites (H60). They showed that the nature of the separation could be changed simply by changing the eluent cation.

Suppressor Technology and Detection. New detectors for IC are not included in this review, the reader is referred to the LC Equipment and Instrumentation review also in this issue.

For ultimate detection limits, suppressed IC is generally superior to single-column or nonsuppressed IC. Midgely and Parker published a very important paper discussing nonlinearity of calibration in the determination of anions by IC with suppressed conductivity detection (H61). They note that curvature may occur in the calibration curve from displacement of eluent ions by the determinand with consequent reequilibration of the conjugate acid in the suppressed eluate. This paper should be read by all those using suppressed IC for quantitative determinations.

Berglund and Dasgupta reported a very clever two-dimensional conductometric scheme for suppressed IC which greatly increases the sensitivity for weak acids which may be hidden in the suppressed baseline or overlapped with strong acid peaks (H62). Strong et al. reported the use of electro-dialytic eluent generation and suppression (H63). This scheme reduces the suppressed conductivity due to residual acids originating from the anionic impurities in the eluent sodium hydroxide and eliminates the conductivity contribution from chemical regenerant penetration in the suppressor.

Shintani and Dasgupta reported a scheme for using pH detection by modifying the pH of the eluent between the suppressor and the pH detector (H64). Obrezkov et al. reported a clever chemical based detector which uses the decomposition of KBrO₃ in HCl in the presence of methyl orange (H65). This reaction is catalyzed by reducing anions and can be monitored at 522 nm.

I. SECONDARY CHEMICAL EQUILIBRIA

The investigation and utilization of secondary chemical equilibria (SCE) such acid-base, complexation, ion-pairing, and solute-micelle associations for modern LC continued in this review period, with many reports concerned with the simultaneous use of two or more phenomena.

Reviews and General Theory. Brinkman and Irth discussed the principles of ligand exchange for trace enrichment and selective detection of ionic compounds (I1). Koenigbauer described applications of micellar mobile phases for the assay

of drugs in biological fluids (12). Werner reviewed the analysis of nucleotides, nucleosides, nucleobases in cells by ion-pair RPLC, including its advantages over anion-exchange and conventional RPLC (13).

Poppe utilized coupled transport equations to mathematically describe secondary equilibria and their interaction with chromatographic transport (14). Solution of the linearized forms of these equations predicts the existence of N eigenpeaks corresponding to the number of degrees of freedom needed to describe a given solution composition, and facilitates the understanding of indirect detection. Knox and Shibukawa showed the effect of the kinetics of the secondary equilibrium process on peak shape and efficiency, using the anomalous bandspreading of ethylenediaminetetraacetochromium(III) ion as an example (15).

Micellar Liquid Chromatography (MLC)

Several thermodynamic studies were reported. Tomasella and co-workers developed a thermodynamic model of MLC that permits the enthalpy of transfer from the bulk aqueous phase to either the micelle or the stationary phase to be measured (16). Their experimental results suggest that as the hydrophobicity of a solute becomes very large, the direct transfer of the solute from the micellar pseudophase to the surfactant modified stationary phase becomes more likely. Subsequent studies (17) showed that direct transfers are much less likely in organic solvent-modified micellar systems because the solute partitioning equilibria are shifted away from the stationary and micellar phases, and toward the bulk hydro-organic phase. Based on comparisons of the retention of 21 aromatic compounds in RPLC with hydroorganic and micellar mobile phases, Lavine et al. (18) showed through correlation analysis that solute retention in MLC is influenced by the net surface charge of the surfactant-modified stationary phase.

Other studies were principally concerned with measurement of solute-micelle association (binding) constants, K_{am} . Garcia et al. (19) measured K_{am} for benzene and naphthalene derivatives with sodium dodecylsulfate (SDS) and hexyltrimethylammonium bromide (HTAB) in the presence of butanol and sodium chloride, whereas Marina et al. (110) measured K_{am} for similar compounds using SDS, cetyltrimethylammonium bromide (CTAB), and polyoxyethylene [23] dodecyl ether (Brij-35). Kord et al. compared the measurement of K_{am} by MLC and micellar electrokinetic capillary chromatography (MECC) and found neither technique to be universally superior (111), with hydrophobic compounds presenting problems for both. In MLC the uncertainty in K_{am} was linked to errors in the CMC and P_{sw} , a constant related to the stationary phase; in MECC the errors resulted from negligible differences in migration times of the solute and micellar phases. Finally, Foley reported values of K_{am} 's for over 150 combinations of solute/surfactant/stationary phase and described theories based on these constants for the optimization of surfactant concentration in MLC and MECC (112).

Using the Knox equation to investigate the causes of reduced efficiency in MLC, Berthod et al. found that flow anisotropy (A term) is the largest contributor, although significant contributions to band broadening from the B and C terms were also observed (113). Hinze et al. (114) characterized and evaluated three different types of chiral surfactant organized media as mobile phases in MLC: bile salts (115), mixed surfactant systems containing a chiral additive, and a chiral quaternary ammonium ionene polyelectrolyte. The bile salts worked well with substituted binaphthyl enantiomers whereas the mixed SDS/chiral nonionic surfactant system was effective with derivatized amino acid enantiomers and anomeric saccharides.

The potential of MLC for QSAR/QSRR has begun to be appreciated. Breyer et al. successfully predicted the biological activity of 26 para-substituted phenols using only one parameter in MLC, the retention factor (k'); in contrast, three conventional descriptors ($\log P_{ow}$, pK_a , and R) were needed to achieve similar success (116). Lavine et al. showed that $\log k'$ for 22 mono-, di-, and trisubstituted benzenes measured on a liquid crystalline stationary phase could be substituted for $\log P_{ow}$ as a measure of hydrophobicity (117).

Love and Fett optimized selectivity via pH and type of surfactant for the determination of drugs in urine by direct

injection (118). Strasters et al. performed a simultaneous optimization of the concentration of surfactant and organic solvent to maximize selectivity for phenolic compounds and a resolution based criterion for amino acids and small peptides (119).

Brando et al. (119a) examined the factors that influenced the retention of inositol phosphate positional isomers on a reversed-phase column with a micellar mobile phase of hexyltrimethylammonium hydroxide (HTAH), including the concentration of HTAH, pH of the bulk micelle suspension, and the addition of inorganic salts to the mobile phase. Finally, Yuan and Wang described the reversed-phase ion-pair chromatography of metal-3,5-diBr-PADAP [2-[(3,5-dibromo-2-pyridyl)azo]-5-(diethylamino)phenol]triton X-100 complexes (120).

Cyclodextrin-Mediated Separations

The number of separations using some type of cyclodextrin as a mobile-phase additive has increased dramatically. For reasons not fully appreciated, the efficiency of such separations significantly exceeds those employing cyclodextrins as a stationary-phase component.

The utility of cyclodextrins as mobile phase additives is sometimes limited by their solubility. Taghvaei and Stewart measured the solubility of and the nitroaniline selectivity provided by the β -cyclodextrin in aqueous solutions of acetonitrile, methanol, dimethyl sulfoxide, and dimethylformamide (121). Addition of up to 20% acetonitrile and methanol resulted in a significant increase (+107%) and decrease in solubility (-60%), respectively. Shimada et al. studied the effect of acetonitrile, methanol, and tetrahydrofuran in the mobile phase on the separation of bile acids and their fluorescent derivatives. Whereas acetonitrile and methanol were equally effective in the separation of free bile acids, the latter was more effective for the 3-(1-anthroyl) bile acids or bile acid 24-pyrenacyl esters (122). Munoz de la Pena et al. studied β -cyclodextrin-pyrene complexes under RPLC conditions with significant amounts of methanol or ethanol present as mobile-phase comodifiers (123). Pawlowska and Lipkowski developed an enantiomeric ternary mobile-phase system of water-ethanol-2,2,4-trimethylpentane with permethylated β -cyclodextrin as a chiral additive (124).

Gosselet and Seville (125) described the effects of β -cyclodextrin in the mobile phase on the retention and indirect detection of aliphatic alcohols. A similar study of two steroids, pregnanolone and progesterone, was conducted by Agnus et al. (126); apparent formation constants of the inclusion complexes were also calculated. Shimada and Nonaka demonstrated the utility of cyclodextrin for the HPLC separation of C21 steroids (127). Lamparczyk et al. measured the inclusion complex formation of some polycyclic aromatic hydrocarbons with β -cyclodextrin (128), and both Jang et al. (129) and Landau and Grushka (130) conducted general chromatographic studies of some cyclodextrin complexes. Finally, Miyashita and Yamashita (131) used a combination of inclusion complex formation and ion pairing for the determination of iodide by HPLC; β -cyclodextrin was found to be superior to α -, γ -, and dimethyl- β -cyclodextrin.

Mohseni and Hurtubise compared the retention characteristics of polycyclic aromatic hydrocarbons, nitrogen heterocycles, and hydroxyl aromatics in RPLC using an aqueous mobile phase with β -cyclodextrin versus MeOH-water and EtOH-water. Later they more closely examined the separation of selected hydroxyl aromatics using β -cyclodextrin (132) and measured changes in the enthalpy and entropy of transfer (133).

Seno et al. reported the chromatographic behavior of α -, β -, and γ -cyclodextrin inclusion complexes of NADH and NADP, and showed that the β -cyclodextrin inclusion complex was the most stable (134).

Ion Pairing

Separations utilizing ion pairing continued to increase in number and complexity during this review period. For the sake of organization, papers in this section are grouped into two categories: simple ion pairing/interaction with no systematic exploitation of other secondary equilibria, and complex systems that utilize ion pairing in conjunction with

complexation, etc. Most of the theoretical papers were concerned with the first category.

"Simple" Ion Pairing. In a series of papers, Bartha and co-workers reported a basis for the rational selection of the hydrophobicity and concentration of the ion-pairing reagent (135), extended the electrostatic retention model of reversed-phase ion-pair chromatography to include the simultaneous effect of the organic modifier and the pairing ion (136) or the effect of pH (137), and developed a practical equation ($\log k' = a + b \log [\text{IP}]$) to predict the effect on retention of the pairing ion concentration (138).

Eppert and Liebscher described factors influencing the resolution of positionally isomeric alkane monosulfonates in ion-interaction RPLC with indirect photometric detection (139). Zou et al. showed that the effect of methanol concentration on retention was greater in ion-pairing RPLC than in conventional RPLC (140). Hansen and Tjørneilund used the highly hydrophobic ion 3-(*N,N*-dimethylpalmitoylammonio)propanesulfonate to dynamically modify silica for ion-interaction separations (141). Callahan et al. used aliphatic diamines as ion-pair reagents to resolve 3',5'- and 2',5'-nucleotidyl diphosphates (142); conventional monoprotic IP reagents yielded no separation. Fornstedt et al. studied the regulation of system peak gradient retention for obtaining analyte peak compression (143) and showed how it can be optimized (144). Okafo and Camilleri compared the separation of dansylated amino acids using deuterium oxide and an ion-pairing agent versus the same system with regular water. The lower retention of the majority of solutes can be explained by the systematically higher pK_a values of these amino acids in deuterated water (145). Patthy et al. studied the effects of the concentration of the pairing ion, organic solvent, buffer salt, and pH on the retention and resolution of myo-inositol phosphates and selected nucleotides and sugar phosphates (146). Shayman and Barcelon synthesized a novel counterion, *N*-methylmipramine, for the separation of similar compounds (147). Finally, Helboe examined the effects of several pair of counter-ions of opposite charge to control the retention of clopenithol and other basic drug substances (148).

Other important separations achieved by "simple" ion pairing included the following: D-myo-1,2,6-inositol triphosphate (149); malondialdehyde, ascorbic acid, and adenine nucleotide derivatives from biological samples using tetrabutylammonium ion (150); a complete set of basic, diastereomeric pentapeptides using 1-pentanesulfonate (151); and nucleosides and nucleotides by two-dimensional ion pairing on polymeric and silica stationary-phase supports (152).

Complex Ion-Pairing Systems. Numerous reports were concerned with the separation of metal ions by complexation and ion-pairing chromatography (typically with TBA). Kaneko et al. determined aluminum in the human serum of a dialysis patient by complexation with 2,2'-dihydroxyazobenzene (153), whereas Uehara et al. employed salicylaldehyde benzoylhydrazone (154). Other researchers were concerned with ions of the transition metals or rare earth elements. Connor et al. speciated iron(II) and iron(III) as their respective 1,10-phenanthroline and 5-sulfosalicylate complexes (155). In contrast, Haddad and Kalambaheti used cyanide as the ligand and TBA as the pairing reagent in their determination of microgram L-1 levels of iron, copper, cobalt, nickel, and chromium (156). Uehara and co-workers determined Ti(IV) in river water using 4,4'-diantipyrilmethane as the complexing agent (157). Other separations of main group and transition metal ions [e.g., Al(III), Bi(III), Fe(III), Co(III), Cr(III), Ni(II), Cu(II), Zn(II), V(V), and Mo(VI)] utilized a variety of complexing agents, including derivatives of 2,2'-dihydroxyazobenzene (158), 2-(3,5-dibromo-2-pyridylazo)-5-[*N*-ethyl-*N*-(3-sulfopropyl)amino]phenol and analogues (159), EDTA (160), *meso*-tetrakis(3-bromo-4-sulfophenyl)porphine (161), 1- or 2-nitroso-2-naphthol-6-sulfonate (162), 2-(3,5-dibromo-2-pyridylazo)diethylaminophenol (163), 4-(2-pyridylazo)resorcinol (Nb(V) and Ta(V) only) (164), the trisodium salt of 2-(*p*-sulfophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid (SPADNS) (165), 4-(2-thiazolylazo)resorcinol (166), and 1-(*p*-nitrophenyl)ethylenediamine-*N,N,N',N'*-tetraacetic acid (167). The last three complexing agents represent new introductions.

Separations of rare earth ions by procedures similar to those for the transition metal ions above were reported using several different ligands (and ion-pair reagents), including lactic acid

Miscellaneous

Several researchers studied effects of acid-base equilibria in conjunction with other chromatographic properties. Although pH effects on chromatographic separations are usually very well understood (qualitatively at least), there are nevertheless some often-overlooked subtleties which can be exploited for improved selectivity and resolution. Little et al. showed that the sequential application of a pH gradient and a solvent gradient in RPLC could be used to separate ionizable compounds such as peptides, phenols, and substituted benzoic acids from neutral compounds of comparable molecular weight (172); the elution of the ionizables during the pH gradient resulted in unique selectivities. Schoenmakers et al. conducted a thorough, systematic study of the effects of pH on retention and selectivity in RPLC, and concluded that pH is somewhat more difficult to optimize than solvent composition due to the former's secondary effects on efficiency and peak asymmetry (173). Tanaka et al. (174) were able to separate nitrozo and oxygen isotopes of amines, carboxylic acids, and phenols by exploiting the isotope effects on the dissociation constants of these compounds (using a mobile phase pH near the pK_a); similar separations of deuterated benzoic acid isotopomers were reported by Lockley (175). By analyzing the dependence of retention on pH in RPLC, Rittich and Pirochtova measured the limiting capacity factors ($\log k'_{\text{neutral}}$ and $\log k'_{\text{anion}}$) of several aromatic acids in RPLC and correlated $\log k'_n$ with $\log P_{ow}$ and $\log k'_a$ with fungicidal activity (176). Yamakawa et al. studied the effects of pH and sodium chloride on the retention and selectivity of transfer ribonucleic acids on a spherical hydroxyapatite stationary phase (177). Finally, Berry and Pretorius described a novel, in-line approach to the preparation of ultra-pure buffered eluents of fixed pH from 2 to 13 (178).

In contrast to the above ion-pairing/complexation systems, many transition metals can also be separated as relatively hydrophobic complexes in conventional RPLC without ion pairing. This approach was employed by Dilli et al. (179), Ichinoki and Yamazaki (180), Fernandez et al. (181), maslowska and Starzynski (182), Main and Fritz (183), Tong et al. (184), and Park and Hardy (185) for the separation of main group and/or transition metal ions using diethyl dithiocarbonate, benzoylacetone, *o*-phenanthroline, acetylacetone, 2-acetylpyridine-4-ethylthiosemicarbazone, 1-phenyl-3-methyl-4-benzoyl-5-pyrazolone, and benzylpropionitrile di-thiocarbamate, respectively, as the complexing agents.

The separations of a number of compounds by ligand exchange were reported, including carbohydrates (186), enantiomers of carbidopa and methyldopa (187), fluorinated metal β -diketone chelates (188), and racemic (189) and chiral amino acid derivatives (190-193). Koziol and Grayeski investigated the ligand-exchange retention mechanism of a novel "triamine" stationary phase made by bonding trimethoxysilylpropyldiethylenetriamine to silica (194).

J. GEOMETRIC AND OPTICAL ISOMERS

Our literature search turned up over 200 original papers on optical isomer separations. It is beyond the scope of this review to include all of these references, and thus we limit our discussion to the more fundamental ones. Fortunately, numerous reviews were published which will alert the reader to most of the articles that we were forced to exclude.

Reviews. Four broad reviews were published during this review period. Enantiomeric derivatization was reviewed by Gorog (J1), whereas the direct separation of drug enantiomers by a variety of methods was the subject of reviews by Gübitz (J2), Mehta (J3), and Subert et al. (J4).

A number of more specific reviews were also reported, with many focused on the type of chiral stationary phase (CSP) employed. Aboul-Enein and Islam reported on structural factors and functional group selectivity requirements for resolution of drug racemates on derivatized cellulose, and presented a guide for the selection of the appropriate chiral derivatized cellulose column based on the elements of asymmetry and functional groups of the compounds (J5). Allenmark reviewed the separations that could be achieved by

immobilizing proteins on reversed-phase chromatographic supports (J6). Armstrong et al. reviewed the applications of (*R*)-naphthylethylcarbamate-derivatized and (*S*)-naphthylethylcarbamate-derivatized β -cyclodextrins. These multimodal phases act as (i) π -complex hydrogen-bonding chiral stationary phases in the normal-phase mode; (ii) unique cyclodextrin phases in the reversed-phase mode; and (iii) cellulosic-like phases when used with methanol, acetonitrile, ethanol, and other organic solvents (J7). Clark reviewed the separation of steroid isomers on porous graphitic carbon using chiral mobile phase additives (J8). Dobashi et al. summarized an approach for enantiomeric separations based on hydrogen-bond association (J9). Duncan compared the separations attained in normal phase HPLC and TLC involving *N*-carbobenzoyloxy-glycyl-L-proline and (\pm)-10-camphorsulfonic acid as mobile-phase additives/countersions (J10). Hansen et al. summarized the use of enzymes as excitatory amino acid receptor agonists for enantiomeric resolution (J11). Isaksson et al. reported the physical and chromatographic properties of microcrystalline triacetylcellulose and reviewed its applications as a chiral stationary phase (J12). Menges and Armstrong gave an extensive discussion of native and functionalized cyclodextrin-bonded phases in terms of their retention and chiral recognition mechanisms, including structure-selectivity relationships (J13). Meyer described the separation of amino acid racemates and use of the relative quantities of enantiomers as a means of dating samples (J14). Okamoto et al. provided two reviews of chiral separations based on polymeric and polymer-coated stationary phases (J15, J16), including polysaccharide carbamate phases (J16). Perrin and Pirkle reviewed commercially available brush-type CSPs (J16a), whereas Wainer et al. reviewed chiral recognition on biopolymer-based CSPs, and presented a case for multiple interaction sites (J17). Lastly, two in-depth reviews of chiral-based separations from a compound-perspective were published. Brash and Hawkins summarized methodologies that have been successful for eicosanoids (J18), and Takagi did likewise for chiral lipid derivatives (J19).

Finally, several authors provided overviews that may be helpful to the novice. The most general outlook was provided by Ahuja (J20). Camilleri surveyed the biomedical applications of chiral liquid chromatography, outlining the following methods for resolution of enantiomers by HPLC: the formation of diastereomers, use of chiral stationary phases, and chiral mobile phase additives (J21). Ley et al. also surveyed these topics along with chiroptical detection from the perspective of pharmaceutical quality control, bioanalysis, and pharmacokinetics (J22), providing an analytical chemist's perspective of drug development (J23).

New Chiral Stationary Phases (CSPs). Many new CSPs were synthesized since the last review, joining the legions of CSPs already developed. The question 'Do we really need such a multitude of CSPs or can we design twenty or fewer that have broad applicability?' that was asked in the previous review does not appear to have been definitively answered. Instead, several dozens of apparently new CSPs have been reported.

Immobilized enzymes continue to be exploited. By activating the stationary phase with 1,1-carboxyldiimidazole, Domenici et al. were able to covalently immobilize human serum albumin (HSA) on a commercial diol column (J24), show that solute retention (k') accurately reflects binding to native HSA (J25), and detect allosteric interaction between warfarin and benzodiazepine binding sites (J26). Erlandsson and Nilsson cleaved and immobilized a fragment of bovine serum albumin (BSA) and showed that its breadth of enantioselectivity was reduced relative to whole BSA; enantiomers of oxazepam, benzoic acid, and morpholep were resolvable whereas tryptophan and warfarin could not (J27). Theolhan et al. immobilized trypsin on a column and were able to separate free and derivatized amino acids that are natural substrates of the enzyme. Jadaud and Wainer resolved the stereoisomers of *N*- α -aspartyl-phenylalanine 1-methyl ester on an immobilized α -chymotrypsin HPLC column and described the effect of mobile-phase composition and enzyme activity on retention (J28).

Although potent in their native form, derivatives of the cyclodextrins have also proven to be useful. Armstrong and co-workers synthesized several derivatized cyclodextrins and reported the first normal-phase separation of enantiomers with cyclodextrin-based media. Stalcup et al. bonded (*S*)-2-hydroxypropyl- β -cyclodextrin to silica gel and successfully separated more than a dozen racemates that had not yet been successfully separated on underivatized β -cyclodextrin. Pawlowska used permethylated β -cyclodextrin in a mobile phase of hexane and ethanol to dynamically coat a conventional silica column for the separation of a variety of enantiomers (J29).

Fischer et al. used the molecular imprinting method (polymerization in the presence of a print molecule) with methacrylic acid and itaconic acid monomer to synthesize the corresponding CSPs for the separation of beta-adrenergic blocking agents (J30). Okamoto and Kaida synthesized and evaluated more than 20 different tris(phenylcarbamate)s of cellulose and amylose and observed the largest enantioselectivity with the 3,5-dimethylphenyl derivatives (J16). Pirkle and co-authors reported new CSPs based on α -amino phosphonate (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine phase (J33). The latter is markedly superior for enantiomers of anilides derived from carboxylic acids. Salle et al. designed and synthesized a new CSP containing a benz[de]isoquinolinone skeleton that showed high enantioselectivity toward 3,5-dinitrobenzoyl- α -amino esters and - α -amino acids (J34). Uray and Linder covalently bonded (*S,S*)-*N*-3,5-dinitrobenzoyl-1,2-diphenylethane-1,2-diamine to silica gel via an undecenoyl spacer and achieved a broad range of enantioselectivity (J35). Oliveros et al. prepared and evaluated CSPs with π -donor and π -acceptor characteristics by bonding the two chiral selectors onto the same silica and by mixing two monofunctional chiral silicas (J36). Finally, Tamai et al. synthesized optically active polyamides having an axially disymmetric 1,1'-binaphthalene-2,2'-dicarboxylic acid component and evaluated their optical resolution ability (J37).

Mobile-Phase Additives/Derivatization. Hinze et al. (J38) characterized and evaluated three different types of chiral surfactant organized media as mobile phases in micellar liquid chromatography: bile salts (J39), mixed surfactant systems containing a chiral additive, and a chiral quaternary ammonium ionene polyelectrolyte. The bile salts worked well with substituted binaphthyl enantiomers whereas the mixed SDS/chiral nonionic surfactant system was effective with derivatized amino acid enantiomers and anomeric saccharides. Walhagen and Edholm showed that the nature of the achiral stationary phase is of little importance when separating enantiomers using β -cyclodextrin in the mobile phase (J40).

The derivatization of enantiomers with a chiral reagent, followed by the separation of the resulting diastereomers on an achiral column, is one viable (albeit sometimes tedious) approach for the separation of optical isomers. Along those lines several new derivatization agents were reported: tartaric acid derivatives for amino-alcohols (J41); *N*-(*p*-toluenesulfonyl)propyl isocyanate for amine and alcohol drugs (J42); fluorescent (*S*)-(-)-2-[4-(1-aminoethyl)naphthyl]-6-methoxy-*N*-methyl-2*H*-benzotriazolyl-5-amine dihydrochloride for carboxylic acids (J43); Sar-L-Phe-OMe and Gly-L-Phe-OMe for *N*-(benzyloxycarbonyl) amino acids (J44); and chiral variants of Sanger's reagent (J45), new chiral thiols (together with *o*-phthalaldehyde) (J46), *N,N'*-bis[(*S*)-1-phenylethyl]carbodiimide (J47), (*S*)-(-)-*N*-(2-naphthylsulfonyl)-2-pyrrolidinecarbonyl chloride (J48), 1-fluoro-2,4-dinitro-5-L-alanine (J49), and benzoylcarbonyl-L-valine- α -aminoisobutyric acid-glycine-*N*-oxysuccinimide for α - and β -amino acids (J50) for free α - and β -amino acids and other related derivatives.

Theory/Mechanisms. A variety of CSPs were examined further in pursuit of a clearer understanding of the mechanism of enantiomeric separation (chiral recognition) and other factors. Importantly, the mechanism was not always the same for all classes of enantiomers on a given column. Dhanesar discussed the influence of mobile and π -donor stationary phases on the separation of racemic 3,5-dinitrobenzoyl-phenylethyl (J51). Berthod et al. reported on the subtleties of interaction of 40 chiral molecules with an (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine CSP (J52). Hu and Ziffer reported a new model to account for the elution order of alkylaryl-

carbinol enantiomers separated on a Pirkle column (J53). Pirkle and Readnour described a chromatographic approach to the measurement of the interstrand distance for some CSPs (J54). Saigo et al. observed odd-even discrimination in chiral recognition ability of optically active polyamides consisting of anti head-to-head coumarin dimer and α,ω -alkanediamine (J55). Udvarhelyi and Watkins investigated the effect of pH, temperature, and mobile-phase additive on the separation of a series of 12 amino acids of the phenylglycine family using a chiral crown ether CSP (J56). Other CSPs studied included the following: (*R*)- and (*S*)-1-(α -naphthyl)ethylamine and derivatives of urea (J57); four π -acid CSPs derived from (*S*)-N-(3,5-dinitrobenzoyl)tyrosine (J58, J59); and natural and chemically modified β - and γ -cyclodextrins (J60-J63).

Two interesting effects of temperature were reported. Gilpin et al. studied the effect of temperature and pH on the chiral recognition of tryptophan by silica-immobilized bovine serum albumin (J64); the discontinuity in the van't Hoff plot was attributed to a phenomenological change in the bound protein similar to a phase transition. Second, whereas retention usually decreases with increasing temperature, Pirkle noted the opposite for selected enantiomers on a CSP (J65).

Three chromatographic phenomena that normally receive little attention in chiral separations were examined during this review period. Pirkle and co-authors found 1,3,5-tri-*tert*-butylbenzene to be a convenient void volume marker under normal phase conditions for three chiral HPLC columns (J66); they also showed that end-capping typically increases the enantioselectivity by reducing silanophilic interactions, particularly on phases with low surface coverage (J67). Rizzi compared the efficiency of chiral separations based on ligand exchange in the mobile or stationary phase as a function of complexation process, flow rate, and capacity factor (J68); the efficiency of the mobile phase additive approach was much higher due to a smaller contribution of ligand-exchange kinetics to the overall plate height. Hargitai et al. immobilized chiral poly(*N*-acryloyl-*S*-phenylalanine ethyl ester) and observed a much improved chromatographic performance with minimal loss in selectivity (J69).

Geometrical Isomers. Relative to enantiomers, geometrical isomer separations received much less attention by researchers. Nevertheless, several noteworthy papers were published. Weber and Carr showed that carbon-clad microporous zirconia exhibits much greater selectivity for structural isomers than conventional RPLC supports, particularly when the isomers incorporate a polar functional group attached to an aromatic ring (J70). Harino et al. showed that both electrostatic and hydrophobic interactions are important in the separation of polar benzene derivatives on poly(vinylbenzo-18-crown-6)-immobilized silica (J71). Klein and Springer reported the first separations utilizing thermotropic liquid crystalline side group polymers coated on silica gel (J72, J73). Retention mechanisms appeared to be different for the steroids and nitrobenzene isomers employed as probes. Paleologou et al. examined the effect of mobile phase composition, pH, temperature, ionic strength, and elution mode (isocratic vs gradient) on the retention behavior and separation of 19 monoaromatic chlorophenols on β -cyclodextrin (J74, J75).

Sander and co-workers investigated the shape discrimination of both electron-acceptor and electron-donor using polycyclic aromatic hydrocarbons (PAHs), methyl-substituted PAHs, and polychlorinated biphenyl congeners (J76); planar compounds were always more retained than corresponding nonplanar analogues. Whereas the addition of methyl groups usually results in an increase in retention in RPLC, Wise et al. observed that this is not the case for PAHs when the methyl groups induce a nonplanar distortion of the molecule (J77). Sato et al. used a cross-linked polyacrylamide gel and normal phase eluents to separate stereoisomers of poly(methyl methacrylate) (J78). Seeman et al. (J79) observed a strong dependence of retention and efficiency on pH for the separation of nicotine and 13 related homologous/isomeric alkaloids on β -cyclodextrin; in contrast the selectivity did not change significantly.

The use of subambient temperatures for enhanced shape selectivity in RPLC was exploited for the separation of epimeric pairs of C-24 alkylsterols (J80) and *cis-trans* isomers of cyclohexanedimethanoldibenzoate using subambient high-performance liquid chromatography (J81). Sentell and

K. MULTIDIMENSIONAL CHROMATOGRAPHY AND COLUMN SWITCHING

As in the last Fundamental Review, multidimensional chromatography will refer only to systems in which the second and any subsequent columns are operated in a different "mode" than the first. That is, true multidimensional chromatography involves a change in retention mechanism between the first and second, third, etc. columns. One of the most rapidly growing multidimensional techniques involves the coupling of LC and GC, particularly LC microcolumns and capillary GC columns, and reports on that topic are included in this review. Column switching will here imply multiple-column systems that involve no retention mode changes, and can be thought of as stationary-phase programming. Although both multidimensional chromatography and column switching can be accomplished off-line, on-line techniques are much preferred because of greater accuracy and precision, decreased analysis times, and amenability to automation. For those reasons only on-line techniques are reviewed here.

A number of reviews addressing various aspects of multidimensional chromatography, including specific applications of the technique, appeared during the past 2 years. Giddings re-emphasized the need to couple orthogonal separation mechanisms to achieve multidimensional separations and used both two-dimensional planar chromatography and coupled columns as examples (K1). Rothman emphasized hardware considerations in automated multidimensional chromatography (K2). Application reviews focused on purification of proteins (K3), pharmaceutical analyses (K4), and pesticide residue analyses (K5). Koenigbauer and Majors discussed the future of sample cleanup using on-line multidimensional LC (K6).

The coupling of achiral and chiral columns proved to be one of the most popular forms of two-dimensional LC during the past two years. Frequently, an achiral first column is used to separate enantiomeric pairs from other matrix components, with the enantiomers then transferred to a second chiral column for selective separation. Such an approach usually enhances the chiral separation by excluding interfering substances from the second analytical column. Stalcup et al. (K7) determined the optical purity of scopolamine derived from *Datura sanguinea* in this way, using a C_{18} column to isolate scopolamine from other alkaloids, then transferring the unresolved scopolamine enantiomers to an acetylated cyclodextrin column via a six-port switching valve. Rizzi coupled achiral ODS columns with a chiral column made of swollen microcrystalline cellulose triacetate (K8). The two-dimensional system is able to compensate for the low peak capacities of swollen cellulose triacetate columns and allows resolution of enantiomers in much more complex matrices than before. Oda and co-workers developed an assay for enantiomers of verapamil and its three metabolites using an achiral ODS column coupled to an ovomucoid protein chiral stationary phase (K9). Verapamil and its metabolites were isolated on the reversed-phase column, selectively switched into sampling loops, and then successively transferred to a trapping column. After dilution with the new mobile phase, each enantiomeric pair was passed to the ovomucoid column and resolved. An ovomucoid column was also used by Tamai et al. in a two-dimensional separation of propranolol enantiomers (K10). The propranolol enantiomers were isolated directly from whole blood, plasma, or tissue homogenates with a short reversed-phase precolumn and then transferred to the chiral ovomucoid column. Finally, in an interesting variation on this coupling of achiral/chiral separation modes, Walhagen and Edholm coupled two achiral columns but added chiral β -cyclodextrin only to the mobile phase in the second column to achieve the same two-dimensional effect (K11). This technique was used to separate enantiomers of chlorthalidone and terbutaline in biological fluids.

One of the most exciting developments in multidimensional separations involved the coupling of liquid chromatography and capillary electrophoresis. Bushey and Jorgenson first reported on the use of such a system involving capillary reversed phase LC followed by CE to compare the tryptic digest

fingerprints of horse heart cytochrome c and bovine heart cytochrome c (K12). They then described a total system in which effluent from the micro RP-LC column is transferred to the CE column via a computer-controlled six-port valve (K13). Debets et al. designed a special valve for coupling CE to HPLC (K14). The valve was tested using quinidine and desipramine in biological matrices as model compounds, and the coupled CE/LC yielded detection limits in the same range as direct injections with precolumns instead of CE. Yamamoto et al. coupled high-performance gel permeation to capillary electrophoresis (K15). These workers inserted an electromagnetic pinch valve between the gel column and the injection port of the CE device to avoid leakage of electricity to the HPLC.

Other two-dimensional separations involved an anion-exchange/RP-LC couple to separate complex peptide mixtures (K16) and microcolumn cation-exchange/size-exclusion coupling for separating proteins (K17). Both systems are automated, and the greatly enhanced resolving power of two-dimensional systems were demonstrated. A β -cyclodextrin bonded phase was coupled to a C_{18} column to measure benzo[a]pyrene in aviation fuel (K18). Finally, an interface that allows coupling of chromatographic modes which require incompatible mobile phases was described by Lewis and Yordy (K19).

Although simple on-line column switching without change of chromatographic mode does not offer as much resolving power as multidimensional chromatography, it is generally easier to implement and can still offer a number of advantages over single-column techniques. It is a very practical substitute for gradient elution, thus reducing analysis times, and is particularly useful for trace enrichment and sample cleanup. Grosse-Rhode et al. developed a column switching technique for group separations of polycyclic aromatic hydrocarbons (PAHs) and heterocyclic nitrogen-containing PAH (K20). Group separations of PAHs and individual N-PAH separations could be achieved on the first C_{18} -immobilized polystyrene column, and the PAH fraction was then backflushed onto a C_{18} -silica column to separate individual members of PAH groups. Dexamethasone was quantified in bovine liver and muscle tissue using coupled-column normal-phase LC (K21). The sample was eluted from a phenyl column and a heart-cut of the dexamethasone peak diverted to a short silica column. The contents of the silica column were then back-flushed onto a cyanopropyl column which isolated dexamethasone. A reversed-phase column switching system was described for the analysis of ampicillin in biological fluids (K22). Hogendoorn et al. developed a sophisticated computer program to optimize column switching that even corrects for distortion of solvent fronts during step-gradient elution in the first column (K23).

As noted previously, column switching is very useful for trace enrichment and sample cleanup, and a number of reports of novel methods were noted. Posluszny and co-workers developed a system for determining drugs in blood plasma that included on-line micellar cleanup followed by reversed phase analytical separation (K24). Halvax et al. combined on-line flow extraction, precolumn concentration of the organic extract, and then normal-phase LC separation to measure caffeine and zidovudine (K25). A highly selective analysis for peptides containing Arg or Lys at their C-terminus was developed by Ohta et al. (K26). This on-line column switching technique used an anhydrotrypsin-immobilized diol-silica precolumn for selective concentration, followed by a reversed-phase analytical column. Metal chelates of Fe, Cu, and Al in plasma were selectively retained on a reversed-phase precolumn while other endogenous plasma constituents were eluted to waste and then separated with an analytical RP column (K27). Anticonvulsant drugs in serum were determined by injecting whole serum into an internal-surface reversed phase column which preferentially retained the small molecules but not serum macromolecules. Analytical separations were then carried out after transfer to a conventional C_{18} column (K28). Strongly protein-bound compounds were determined in a similar way, using, however, a protein-coated ODS concentrator column (K29). Dialysis was coupled on-line with column-switching HPLC via a C_{18} precolumn concentrator in the analysis of nitrofurans used in veterinary practice (K30). Finally, two techniques which use ion-pairing principles in column switching were reported. In the first (K31), tropane alkaloids were eluted from an ODS pretreat-

ment column with a primary mobile phase that did not contain the ion pair counterion (sodium dodecyl sulfate), transferred to a second ODS analytical column, and then eluted with a second mobile phase that did contain the counterion. In the second application, several nucleosides, including AZT, were first concentrated via ion pairing on a polymeric reversed-phase precolumn and then analyzed by ion-pairing LC.

The coupling of liquid chromatography and capillary gas chromatography provides one of the most powerful two-dimensional separation techniques possible, and the continued development of microcolumn LC is further encouraging applications of this method. Improvements in solvent evaporation and transfer methods continue to appear. Two primary transfer techniques are available: loop transfer and direct transfer using an autosampler as an on-column interface. In partially concurrent solvent evaporation, the LC eluent is not totally evaporated, and the condensed solvent used for solute trapping. In fully concurrent solvent evaporation, the eluent is completely evaporated during transfer. Partially concurrent evaporation allows analysis of components which elute from the GC column at temperatures only a few degrees above that of the transfer line, but requires very stable and reproducible LC eluent and GC carrier gas flow rates. Fully concurrent evaporation requires less critical conditions and allows for transfer of large LC fractions, but a difference of 80–120 °C between elution and transfer temperatures is necessary to prevent peak broadening and distortion.

Cortes and co-workers recognized the advantages of LC microcolumns in coupled LC-GC analyses of pesticide residues (K31). Cortes also combined supercritical fluid extraction, microcolumn LC, and capillary GC in the analysis of trace levels of the insecticide chlorpyrifos in grass samples (K32). Vreuls et al. developed a technique for coupling reversed-phase LC columns to GC that used a short polymeric trapping column (K33). After the mobile phase has been displaced by water, analytes are desorbed with ethyl acetate under partially concurrent solvent evaporation conditions. Schmarr et al. (K34) described an early vapor exit for partially concurrent solvent evaporation that could accept LC flow rates typical of microbore columns (100–200 μ L/min). Dolecka described a system which accelerates solvent evaporation in the GC retention gap (K35). By inserting a split outlet between the retention gap and the analytical capillary column, considerable increases in evaporation rates could be generated. The rates could be varied by varying the back-pressure of the splitter device.

L. PREPARATIVE LC

Theory/Optimization

During this review period, Guiochon's group published a series of papers on optimizing experimental conditions in preparative separations (L1–L7). These studies were concerned with optimization of production rate, as well as with other experimental parameters. Several models used for simulating elution in nonlinear chromatography were compared (L8). A review was presented on the theory of nonlinear preparative chromatography (L9).

Guiochon and El Fallah (L10) investigated the elution profiles of high-concentration bands of a single component during gradient elution. These authors also used a delayed injection procedure to study the interaction of two overloaded bands (L11). Katti and Guiochon (L12, L13) studied the interaction of overloaded binary mixtures and compared these results to a competitive Langmuir isotherm model. Improved predictions were obtained using a hodograph transform method (L14).

A bi-Langmuir isotherm model was used to predict band profiles of two enantiomeric amino acids chromatographed using a chiral stationary phase (L15). Diack and Guiochon (L16) determined the adsorption isotherm of phenyldodecane in acetonitrile onto a carbon packing and used the sum of a quadratic and Langmuir term to describe the isotherm, which exhibited two inflection points.

Dosee and Guiochon (L17) reported on a normalized method to determine overlap between non-Gaussian peaks. Analytical expressions were derived for measuring overlap between Gaussian, Lorentzian, rectangular, right-triangular, and isosceles-triangular peak shapes. This group also investigated the effects of extracolumn components on nonlinear chro-

matographic peak shapes by convoluting column inlet and outlet profiles using simulation models (L18).

Snyder and co-workers (L19) reported on Craig simulations for isocratic preparative LC as a function of retention, column efficiency, and sample size. Each separation was found to have an optimum sample size and column efficiency that are related to the desired recovery of purified product and peak retention. Guiochon (L20) commented on this study and showed that the adsorption isotherm used by Snyder is the cause of the questionable results they obtained. A rejoinder by Snyder and Cox (L21) was also published. In a second paper, Snyder and Cox (L22) derived general equations that relate production rate and run time plus optimum column length and flow rate to maximum operating pressure, particle size, and sample molecular weight. Comments on this study were published by Guiochon (L23) and Snyder (L24). Part three of this series of papers by Snyder and Cox (L25) dealt with two adjacent peaks having unequal column saturation capacities. The practical consequences of variations in solute column capacity were discussed in detail.

A second series of papers, written by Snyder and colleagues, dealt with preparative LC under gradient conditions using the Craig distribution model, which shows that there is a relationship between band width, sample size, and gradient conditions (L26). On the basis of this work, a commercially available computer program was described to aid in the development of preparative separations of peptides and protein samples using reversed-phase gradient elution (L27, L28). Craig simulations for heavily loaded gradient elution were also described in detail (L29, L30).

Jaulmes and Vidal-Madjar (L31) developed a simulation algorithm to study slow kinetic effects in nonlinear LC. This group (L32) also presented a multivalent ion-exchange model to simulate isocratic and gradient elution of biopolymers. Lucy et al. (L33) used several kinetic models to investigate overload processes in reversed-phase LC. More recently, Lucy et al. (L34) studied peak interactions under concave isotherm conditions in preparative ion-exchange LC. Wilhelm and Riba (L35) presented a model for the scale-up and optimization of an LC separation. The model took into account axial dispersion and mass transfer. A theory of nonlinear LC based on mass balance equations was presented by Nowakowski (L36), which considered axial dispersion, flow rate, and mass transfer.

Hearn and colleagues (L37) described a model based on Langmuir adsorption isotherm to predict the performance of bioaffinity columns packed with nonporous particles. Svoboda (L38) developed an equation to describe the mass overload of compounds with S-shaped isotherms. A method for determining the elution profile of a solute of a type II isotherm was presented by Hong (L39).

Frey (L40) presented asymptotic relations for gradient elution of biomolecules. Firoz and Horvath (L41) used computer simulation to study gradient elution at nonlinear conditions. Yamamoto et al. (L42) presented a method for determining stepwise and gradient elution conditions for ion exchange of proteins. Kawasaki (L43, L44) improved upon a general theory of gradient elution and applied it to preparative LC under overload conditions.

Jacobson and Frenz (L45) described two approaches for determining competitive adsorption isotherms from multicomponent frontal LC. Dantigny et al. (L46) reported on an approach for optimizing frontal chromatography. Hill and co-workers (L47, L48) discussed the use and application of frontal chromatography for preparative separations.

Hodges et al. (L49) described a multicolumn approach for preparative reversed-phase sample displacement chromatography of peptides and compared this method to gradient elution LC. Preparative displacement LC was also used for the separation of peptides and proteins (L50–L52).

Colin and colleagues (L53) developed a new concept for improving the cycle time and sample throughput in preparative LC. In this procedure, the direction of the mobile phase is reversed just after the compound of interest is collected. Cretier et al. (L54) studied optimum sample injection conditions for maximizing the recovery of the first eluted peak using two simulation models. Rasmussen and McNair (L55) investigated the effects of sample solvent strength and injection volume on band broadening and throughput in reversed-phase preparative LC. Zogg et al. (L56) reported on

the influence of solvent strength and flow rate, as well as column dimensions, on preparative separations. Under overload conditions, stationary-phase effects that influence the performance of reversed-phase packings were examined (L57). Nicoud and Colin (L58) reviewed the optimization of cost factors in preparative LC.

Input-output models utilizing discrete variables were developed by Frey (L59, L60) and used together with control theory to investigate relationships between output variables (yield, purity, and production rate) and input variables (cut point locations and feed slug size). Kubota and Hayashi (L61) carried out a mathematical analysis of the elution curve in preparative LC with moving feed ports.

Packings/Column/Hardware

A wide-pore (4000-Å) polymer packing consisting of cross-linked polystyrene was developed for preparative HPLC of biopolymers (L62, L63). The packing can be used either for reversed phase or ion exchange after derivatization. A 250-Å pore-size packing of cross-linked poly(methyl acrylate) was introduced and evaluated for the preparative separation of aromatic amino acids (L64). Krause et al. (L65) evaluated the use of polyethylene and polypropylene stationary phases and compared their performance with LiChroprep RP-18. These types of packings proved useful particularly for basic peptides.

The performance characteristics of a number of different ion-exchange packings have been evaluated for preparative separations of proteins. These packings included TSK-DEAE 5PW (L66), Whatman DE52 (L67), CM-Toyopearl (L68), and Q-Sepharose HP (L69). Narayanan et al. (L70) evaluated several silica-based affinity chromatographic packings and found that a pore diameter of at least 200 Å is needed for preparative separations of proteins of molecular weights in excess of 150 000 g/mol.

Zogg et al. (L71) tested various procedures for packing preparative columns with silica of different particle size and particle-size distributions. The authors found that two variations of a dry-packing procedure gave the best packing density. Wang and co-workers (L72) developed a new packing procedure for preparative columns. The procedure consists of adding a slurry of reversed-phase packing and a deflocculating solvent (e.g., acetone) into the column. After the packing sediments, a flocculating solvent (e.g., methanol-water) is then added to solidify the bed.

A new technique for the introduction of insoluble samples in preparative LC was reported by Miller et al. (L73). Porsch et al. (L74) described an intracolumn injection system for large-diameter LC columns.

Kissler (L75) reviewed the advantages of glass columns as compared to stainless-steel for preparative chromatography of natural products. Cox (L76) described the design of fixed-geometry columns, variable-geometry columns, constant compression systems, and manually compressed columns. Colin and co-workers (L77) reported on a dynamic axial compression column design. Patents were awarded for several preparative column designs (L78, L79). A commercial automatic sample processor was described for sample preparation, dilution, injection, column switching, detector feedback control, back-flushing, trace enrichment, and fraction collection (L80).

Selected Applications

A number of review articles on preparative separation of enzymes (L81), proteins (L82–L84), membrane proteins (L85), and enantiomers (L86) have appeared. Preparative-scale recycling HPLC of natural products was reviewed by Kubo and Nakatsu (L87). Desai (L88) presented an overview of the use of immunoaffinity adsorption preparative chromatography.

Specific applications for preparative LC have been reported for albumin (L89), amino acids (L90), apolipoprotein (L91), avidin (L92), carbohydrates (L93) and oligogalacturonic acids (L94), carotenoids (L95, L96), fats (L97), α -fetoprotein (L98), fulvic acid (L99), glycoproteins (L92, L100), herbal plant extracts (L101), hop bitter acids (L102), human growth hormone (L103), immunoglobulin (L89), insulin (L103–L106), monoclonal antibodies (L107), nucleotides (L108), oligonucleotides

M. PRE- AND POSTCOLUMN DERIVATIZATIONS

This section of the review will first list relevant review articles and fundamental studies, and will then group articles according to solute type or functional group. The searches revealed literally hundreds of articles published during this 2 year period. Only those of a more fundamental nature will be included here.

Derivatization is a necessary evil for many chromatographic analyses to add a detectable group, or even a more sensitive detectable group to the solute(s) of interest. Chemistries have been developed for virtually every type of compound, and advances are now coming in the design of faster reactions, more selective reactions, and the synthesis of highly sensitive derivatizing agents. Somewhat surprisingly, most interest in this review period is still in precolumn schemes. Postcolumn derivatization offers inherent advantages over precolumn methods in terms of precision, ease of automation, and the lack of necessity for a single reaction pathway. However, these schemes require more care in the initial design to minimize extracolumn band broadening, and they require reasonably fast reaction kinetics. Many reactions developed initially for flow injection analysis should be easily adaptable to postcolumn reaction detection for liquid chromatography, and the fundamental studies of reactor designs for one area should be of use for the other.

A book in the Dekker Chromatographic Science series, *Detection-Oriented Derivatization Techniques in Liquid Chromatography* was edited by Lingeman and Underberg (M1) and contained several chapters reviewing fundamental areas of derivatization. The editors also authored a chapter in this book reviewing both pre- and postcolumn derivatization techniques in pharmaceutical research (M2). Other general chapters were by Fruijtier et al. (M3) on the role of kinetics in derivatization reactions, and by De Jong et al. (M4) on the theoretical aspects of postcolumn reactors and reaction types. Other more specific chapters were on ultraviolet-visible derivatization (M5), electrochemical (M6), fluorescence (M7), and chemiluminescence derivatization (M8).

The most common method of introduction of the reagent for postcolumn reactions is through a tee connector, but other methods may offer some advantages. Haginaka reviewed the use of membrane reactors as reagent introduction systems and pointed out several advantages including the minimization of band broadening and reduction of sample dilution (M9). Engelhardt and Schoendorf also compared the principles of postcolumn derivatization detection schemes and flow injection analysis (M10). These two methods are somewhat similar, and advances in one area can be easily translated to the other. One area where flow injection has been applied is in the measurement of reaction kinetics. Luque de Castro and Fernandez-Romero described a method of reaction rate measurement using a postcolumn reaction, and applied it to the three main creatine kinase isoenzymes in serum samples (M11).

Other reviews that appeared during this 2-year period were by Krull et al. (M12) on photochemical derivatizations, Ohkura and Nohta reviewed fluorescent derivatization reagents and reactions according to the functional groups of the analytes (M13), and Lunte reviewed both pre- and postcolumn derivatization schemes for generating electrochemically detectable species (M14).

This review period saw further interest in the use of solid-phase derivatizing agents. The use of these agents in place of solution-phase reactions has certain advantages, including in some cases the ability to filter and reuse the derivatizing agent. Krull's group was especially active in this area and reported polymeric agents for weak nucleophiles including primary and secondary alcohols (M15) and for amines, amino alcohols and amino acids (M16). They also combined several different reagents into a single, mixed bed reactor, useful for simultaneously preparing several derivatives from a single analyte (M17).

Other groups were active as well. Jedrzejczak and Gaind described a mixture of silica gel and a polymeric anhydride

containing an *o*-acetylsalicyl group as the labeling moiety which was useful for the simultaneous collection and derivatization of airborne primary aliphatic amines (M18). Yasaka and Tanaka et al. described two different polymeric reagents for fatty acids (M19, M20).

The application of aqueous micellar solutions in derivatization reactions has also been investigated during this review period. Micellar systems can be advantageously used both for solubilization of hydrophobic reagents and also to speed the kinetics of slow reactions. Xia and Cassidy investigated the effect of cationic, anionic and nonionic micellar solutions on the post column derivatization and subsequent detection of various metal ions (M21). Van der Horst et al. studied the use of cationic micelles for the precolumn derivatization of the anti-Parkinson drug amantadine (M22). They found that the use of the micellar solution in place of a purely aqueous solution lowered the required reaction time from 20 to only 4 min! Van der Horst et al. also described a micellar phase-transfer catalysis for the automated determination of free fatty acids in plasma (M23). Here the use of the micellar system greatly reduced sample handling and the reaction of the fatty acids with a fluorophore was complete in 5 min.

The use of postcolumn photolysis to generate fluorophores or chromophores or to increase sensitivity has also received considerable interest in this period. This method, where applicable, is simple, as generally no chemical agents are added. The photons are the derivatizing agent. De Ruiter et al. (M24) dansylated pentachlorophenol precolumn and then used postcolumn photolysis to generate fluorescent products. Kwakman et al. (M25) dansylated alkyl-, nitro-, and chlorophenols and then used postcolumn photolysis to generate products which were detected by peroxyoxalate chemiluminescence. Mattusch et al. (M26) studied the photochemical behavior of triazines and found that different classes of these compounds could be distinguished based on their behavior in a postcolumn photochemical reactor. Nicotinic acid and nicotinamide were determined with postcolumn UV irradiation and fluorescence detection by Mawatari et al. (M27). Dou et al. (M28) showed that electrochemical detection of proteins is possible following photolysis. Patel et al. (M29) examined several classes of nitrogenous pharmaceuticals for fluorescence after UV photolysis and also investigated the reaction of the photolysis products with *o*-phthalaldehyde-2-mercaptoethanol.

Immobilized enzymes would seem to offer an excellent means of producing postcolumn reactions; unfortunately, the concentrations of organic solvents that are generally necessary for separation by either normal or reversed phase chromatography rapidly denature the enzymes. In spite of this limitation, much work has been devoted over the years to the development of separations with mobile phase conditions that are compatible with continued enzyme activity. Marko-Varga and Gorton reviewed the use of immobilized enzyme reactors, especially for use with amperometric electrochemical detection (M30). In this review period new enzyme catalyzed methods were developed for the chemiluminescent detection of free fatty acids (M31) and for the electrochemical detection of biological polyamines (M32).

The increased use of LC/MS for structural identification and for trace analysis has become apparent, as for the first time there has been interest in the use of derivatization reactions for this mode of detection. In an interesting fundamental study, Rakotomanga et al. (M33) used thermospray LC/MS to identify byproducts in phenyl isocyanate precolumn derivatization reactions. Five compounds resulting from the reaction of phenylisocyanate and the reaction medium were identified: two from a reaction between phenyl isocyanate and methanol, two from the reaction between phenyl isocyanate and water, and one from the polymerization of phenyl isocyanate. There were also two reports of derivatizations to enhance either the response or structural information from thermospray LC/MS for linoleic acid lipooxygenase metabolites (M34) and for cortisol (M35). This is likely an area that will receive increasing interest. A similar study was reported showing improved liquid secondary ion mass spectra for derivatized oligosaccharides (M36).

The determination of amino acids is likely the most studied and most heavily used derivatization. There are many classic reactions which yield detectable products. Fuerst et al. compared four precolumn methods for free amino acids, the *o*-

phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC), and 1-(dimethylamino)naphthalene-5-sulfonyl chloride (dansyl-Cl) (M37, M38). They concluded that superior sensitivity favors the use of OPA, FMOC-Cl, and dansyl-Cl techniques, but because of instability of the OPA adducts, automated online derivatization is necessary. Fermo et al. (M39) compared two methods, the OPA and *N,N*-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) reactions, for the determination of serum amino acids. They concluded that the FDNDEA method was more reproducible.

Because of the large numbers of amino acid analyses which are performed, there is considerable interest in the automation of this process, including the derivatization step. During this review period, fully or partially automated amino acid methods were reported for the FMOC reaction (M40), the naphthalenedialdehyde (NDA) reaction (M41), the OPA reaction (M42-M46), and the fluorecamine reaction (M47).

New derivatizing agents were also reported, including 4-nitrophenyl isothiocyanate for UV detection (M48), several fluorescence agents, phthalimidylbenzenesulfonyl chlorides (M49), thiamine after chlorination of peptide bonds with hypochlorite (M50), and 3-benzoyl-2-naphthaldehyde (M51). De Montigny et al. (M52) optimized the NDA reaction for tripeptides and reported a competing nonproductive reaction pathway for this reagent.

There was also significant interest in electrochemical detection of derivatized amino acids. Dou and Krull reported that aromatic and sulfur containing amino acids, peptides and proteins could be determined electrochemically after postcolumn photolytic derivatization. The electrochemical behavior of the more common derivatizing agents was also studied, including the dansyl amino acids (M54), the phenylthiohydantoin (PTH) and methylthiohydantoin (MTH) derivatives (M55) and the PITC derivatives (M56). Ferrocenic derivatizing agents for peptides and proteins were synthesized and studied (M57), as was a postcolumn method based on the classical biuret reagent and containing Cu(II), tartrate, bicarbonate, and base (M58).

Other amine compounds are also very heavily studied because of their biological, industrial and environmental importance. Lunte and Wong reviewed the use of the NDA reaction for the determination of primary amines by fluorescence, chemiluminescence or electrochemical detection (M59). Imai et al. (M60) reviewed the use of fluorogenic reagents having benzofurazan structures which are applicable for amines and thiols. Mentasti et al. (M61) studied the behavior of homologous amines comparing the OPA reaction and 4-chloro-7-nitrobenzofurazan.

There were three reports of new peroxyoxalate chemiluminescent reagents for amines: a 7-aminocoumarin derivative (M62), 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (M63), and naphthalene- and anthracene-2,3-dialdehyde (M64).

New fluorescent agents for amines were also of interest during this review period. These include 1,2-diarylethylenediamines (M65), 1-pyrenaldehyde (M66), 5-(4-pyridyl)-2-thiophenecarbaldehyde (M67), 2-(9-anthryl)ethyl chloroformate (M68), 3-(2-furoyl)quinoline-2-carbaldehyde (M69), three benzofurazan derivatives (M70) and 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (M71).

Two electrochemical derivatization methods were reported for amino compounds: 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (M72) and a tetrathiafulvalene derivative (M73).

Two UV-visible absorbance derivatization reagents were also reported. *N*-Methyl-9-chloroacridinium triflate was used for aromatic amines (M74), and polyamines were determined after reaction with 1-phenylsulfonyl-3,3,3-trifluoropropene (M75).

As well as amino acids and amines, there are many other compounds which lack easily detectable groups. Saccharides are one class in which there is always much interest, and in this review period two new derivatization schemes were reported using benzamide (M76) and FMOC-hydrazine (M77) to produce fluorescent products.

Thiols are also difficult to detect, and several new schemes were reported for these compounds. 4-(*N,N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (M78), aroyl-

acrylic acids (M79) and monobromobimane (M80) were reported as new fluorogenic reagents, and ethacrynic acid (M81) was reported as a new reagent for UV detection.

The derivatization of carboxylic acids also received considerable attention during this review period. Several new fluorescent tags were reported; 4-(bromomethyl)-6,7-(methylenedioxy)coumarin (M82), monodansylcadaverine (M83, M84), *N*-(1-pyrenyl)bromoacetamide (M85), *N*-(9-acridinyl)bromoacetamide (M86), 6,7-dimethoxy-1-methyl-2-(1*H*)-quinoxalinone 3-propionylcarboxylic acid hydrazide (M87), 4-(aminosulfonyl)-2,1,3-benzoxadiazole and 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (M89), which was also reported as a useful UV labeling agent. Another UV tag was also reported, 2-(phthalimino)ethyl trifluoromethanesulfonate (M90).

Steroids are another group of compounds which generated considerable interest. Nozaki et al. reported the determination of several steroids fluorometrically by the addition of H₂SO₄ to serum deproteinized with ethanol (M91). They suggested a pH of 1.85 as the optimum for fluorescence; however, this would have to be performed with a resin-based reversed-phase column, as the lifetime of a silica-based column would only be a few injections. Two other fluorescence agents were also reported: a 1-cyanoisindole derivative (M92) and anthracene-1- and 2-carboxylic acid hydrazides (M93). Ferroceneboronic acid was used to add an electrochemically active tag to brassinosteroids (M94), and 5-(*N,N*-dimethylamino)-naphthalene-1-sulfonylhydrazine was used as a labeling agent for a peroxyoxalate chemiluminescence reaction (M95).

Three new fluorescence agents were reported for alcohols: 2-(phthalimidyl)benzoylazides (M96), 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (M97), and both 1,2-diamino-4,5-dimethoxybenzene and 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (M98).

Fatty acids have often been separated by GC after derivatization, but LC is more and more becoming the method of choice. Derivatization is still necessary, however, to add a detectable functionality. Fluorescent tags are the most popular, and in this review period several new reagents were reported. These include boronomethylmethoxycoumarin (M99), 9-anthryldiazomethane (M100), 2-nitrophenylhydrazides (M101), and naproxen chloride (M102). Benzoylation was reported as a useful UV tagging method (M103).

New reactions and reagents were also developed for a variety of other compounds. In the interest of space, only the analyte group will be mentioned; interested readers should then see the original article for the derivatizing group and conditions. These include nucleotides (M104), fluoropyrimidines (M105, M106), α -keto acids (M107), hyaluronic acid oligomers (M108), sulfa drugs (M109), lipase activity (M110), polycyclic aromatic hydrocarbons (oxidized with Ce(IV) to make them electroactive) (M111), aldehydes and ketones (M112), cycloserine (M113), *N*-methylcarbamoyloximes and *N*-methylcarbamates (M114), nonionic surfactants with ester groups (M115), and phenoxy acid herbicides (M116).

Kieber and Blough developed a very unique method for the determination of carbon centered radicals in aqueous solution (M117, M118). The radicals are trapped by a water soluble amino nitroxide, followed by derivatization with fluorecamine. They reported detection limits of 0.5-2 nm, which is 2-3 orders of magnitude lower than by ESR methods!

There was also significant interest in the pre- and postcolumn derivatization of metal ions and other inorganic species. Neidhard et al. reviewed postcolumn reactions for the determination of alkylated species (M119). Other species determined include mercury, cadmium and zinc by postcolumn derivatization with a water-soluble porphyrin (M120), metal ions by precolumn chelation with Eriochrome red B (M121), arsenate, germanate, phosphate, and silicate determined by a postcolumn reaction with molybdenum blue (M122), cyanide as a 1-benzoyl-1,2-dihydroquinaldonitrile complex (M123) and after reaction to form a polymethine dye (M124), noble metals as their thiazolylazoresorcinol chelates (M125), rare earth elements after reaction with 2-(4-arsenophenylazo)-1,8-dihydroxy-7-(2,6-dibromo-4-fluorophenylazo)naphthalene-3,6-disulfonic acid (M126), and various metals as formazan derivatives (M127).

N. MICROCOLUMN, CAPILLARY, AND OPEN TUBULAR LC

In the 1990 Fundamental Review the term "microbore" was used to describe columns with internal diameters between 0.5 and 2.0 mm, and "microcolumn" referred to columns with internal diameters less than 0.5 mm. Microcolumns were further distinguished as packed capillary (ca. 40–300- μm i.d.) or open tubular capillary (ca. 3–50- μm i.d.). The same terminology will be used this year. However, since no fundamental studies specifically addressing microbore columns were noted during searching of the CA database, they will not be included in this year's review. It should be noted, however, that a significant number of applications of microbore LC did appear during 1990–91. Although microbore columns have not proven to yield significant increases in separation efficiency relative to conventional columns, workers are still being drawn to them because of the much smaller solvent flow rates required. These lower mobile phase flow rates make interfacing microbore columns to many detectors, particularly mass spectrometers, much easier. Microbore columns also have distinct advantages for use in two-dimensional chromatography, and many citations in the *Multidimensional LC and Column Switching* section of this review include a discussion of one or more microbore columns.

Ishii and Takeuchi (N1) discussed the advantages of miniaturization of column liquid chromatography, including increased resolution, decreased solvent consumption, lower heat capacity of columns, increased mass sensitivity, and easier coupling of columns to detectors and secondary chromatography systems. Novotny reviewed the advantages of both microcolumn LC and capillary electrophoresis techniques for structural characterizations of biomolecules (N2). Scott extended the theory of open tubular columns to liquid chromatography without regard to any instrumental limitations (N3). Methods for calculation of optimum column length, radius, and film thickness to provide for minimum analysis time were presented. Wilson et al. (N4) studied the effect of column diameter (d_c) to particle diameter (d_p) ratio in 530- μm microcolumns. Previous studies of this so-called Knox-Parcher ratio in conventional LC columns demonstrated that a minimum in reduced plate height is reached when d_c/d_p is between 4 and 6, and similar effects were observed in packed fused silica capillary columns with diameters between 5 and 20 μm (N5). The study by Wilson and co-workers differed from previous ones in that column diameter was fixed at 530 μm while particle diameter varied from 5 to 120 μm . One of the most interesting results to emerge from this study was that, in 530- μm i.d. microcolumns, 70- μm particles are just as efficient as 40- μm particles. Therefore, when pressure limitations are imposed, 70- μm particles can achieve more than 3 times the absolute efficiency of 40- μm particles by employing longer columns.

Micro LC was compared to both conventional, wide-bore (9.4-mm) size-exclusion LC (N6) and capillary zone electrophoresis (CZE) (N7). Ling et al. (N7) evaluated the utility of micro LC and CZE to separate fluorogenically-derivatized thiols of biological importance. Although CZE in a 20-cm \times 25- μm -coated capillary proved more efficient (300 000–500 000 plates/meter) than micro LC in a 250 \times 0.32-mm-i.d. fused silica column packed with 5- μm RoSIL C18 particles (70 000–80 000 plates/meter), selectivity enhancement was much easier in the micro LC system. Micro LC also generated more reproducible retention times and peak areas than CZE, as well as greater sensitivity due to higher column and concentration loadability. Kennedy and Jorgenson (N6) packed microcolumns of 28- and 50- μm i.d. with 4.5- μm size-exclusion Zorbax particles and compared them with 9.4-mm-i.d. columns packed with the same particles. Using three proteins as test probes, it was found that the microcolumns exhibited more symmetrical peak shapes and lower plate heights. The greater efficiency of the microcolumns was attributed to a significantly lower van Deemter A term.

A number of developments in column technology were reported. Tock et al. (N8) described a procedure for designing open tubular LC columns that are optimized for mass loadability. Their approach suggests that the optimum contribution of the stationary phase mass transfer term to the overall plate height should be approximately 50%, instead of the

previously accepted value of 20%. These workers used this approach to optimize the loading of a 21- μm -i.d. open tubular column, which then showed about the same mass loadability as a similar i.d. micropacked capillary. Poly(methylhydro-siloxane) deactivation of fused silica micropacked capillary columns does not appear to affect chromatographic characteristics but does result in far greater tensile strength under high pressures (N9). Eguchi et al. described an in situ technique for fabricating polymer films inside quartz-glass capillaries that uses chain cross-linking photopolymerization of monomers in solution rather than cross-linking of polymers cast from solution (N10). Two silicone coatings for open tubular LC were evaluated (N11): methylvinylsilicone gum (PS-255) and methylphenylsilicone gum (PS-264). Dicaronylene was found to have planarity recognition capability similar to polymeric octadecylsilica in LC microcolumns (N12). Finally, two kinds of cross-linked chitosan beads (a natural polysaccharide composed of 2-amino-2-deoxy-D-glucose units) were evaluated as stationary phases in micro LC (N13). Aminoalkyl-substituted chitosan behaved similar to the commonly used aminopropyl phase, while phenyl-derivatized chitosan showed planarity recognition capability for PAH similar to monomeric octadecylsilica.

Innovations in microcolumn methods included development and control of pH gradients. Slais and co-workers described the separation of a number of proteins in microcolumns using a sample-induced internal pH gradient (SIG) (N14, N15). This same group also developed an on-line, precolumn photochemical technique for generating pH gradients in microcolumns and applied the technique to the separation of methotrexate from its synthetic impurities (N16). Electrically-driven (ED) open tubular LC was also the studied as an alternative to pressure driven (PD) OT LC (N17). ED-OT LC, also known as capillary electroosmotic chromatography, leads to a smaller contribution to plate height from the mobile-phase mass-transfer term due to the plug flow that is generated. Electroosmotic mobility is apparently little affected by the application of an ODS coating to the capillary tube. ED-OT LC was shown in this study to be approximately twice as efficient as PD-OT LC. Pfeiffer and Yeung demonstrated that electroosmotic flow in ED-OT LC, which is inherently slow and can make many separations impractical, can be enhanced through the use of surfactants (N18).

Improving pumps, injectors, and detectors to meet the stringent requirements of microcolumn and open tubular LC continues to interest several research groups. Jorgenson and co-workers described a dedicated capillary LC instrument that uses an optically controlled sample gating procedure (N19). The efficiency of the instrument was demonstrated through separation of a mixture of fluorescein isothiocyanate labeled amines. The combination of short analysis times and automated sample injection allowed signal averaging of data to enhance quantitative precision. Claessens et al. presented a method for determining injection profiles and used the method to compare two different open tubular LC systems (N20).

A number of improved gradient elution systems designed specifically for microcolumn and open tubular LC were described. Bauer's gradient system consists of a variable volume mixing vessel of either 5–100 or 25–1000 μL and produces highly reproducible gradients in short micropacked and open tubular columns (N21). A multiple-loop valve is at the heart of a system described by Banks and Novotny that produces a step-wise addition of mobile phase at low $\mu\text{L}/\text{min}$ flow rates (N22). An open tube gradient generator was developed by Berry et al. that generates S-shaped microgradients (N23). In this work a wide-bore fused silica generator was used; however, the same system could be used with narrow bore open tubes that should permit submicroliter gradients. The first commercially available micro LC system with automatic gradient elution was evaluated with micropacked capillary columns (N24).

Improved detection techniques that can exploit the inherent advantages of microcolumn LC (e.g. low solvent flow, high mass sensitivity) have been the subject of numerous investigations. Coupling mass spectrometers to micro LC has received particular attention. Moseley et al. developed a continuous-flow FAB/MS interface that uses coaxial introduction of the FAB matrix (N25). Addition of the FAB matrix to the mobile phase is not acceptable in micro LC because the

volume necessary greatly exceeds optimum micro LC flow rates, and postcolumn introduction of matrix causes peak broadening because of the relative magnitudes of the two streams. In the coaxial interface, two separate fused silica capillary columns are used so that mixing of the FAB matrix and LC effluent occurs at the FAB probe tip. The interface was shown to offer 4 times the separation efficiency over precolumn addition of matrix when used with a 320- μm -i.d. packed capillary column (N26). The coaxial, continuous flow FAB interface was also used to couple 50- and 75- μm -i.d. C_{18} -micropacked capillary columns to a tandem mass spectrometer. Daughter-ion spectra of separated peptide mixtures and tryptic digests at the picomole to femtomole level were also reported (N27). The same interface described in the previous three citations was modified to allow for coaxial delivery of sheath fluid for electrospray ionization MS (N28). Postcolumn addition of FAB matrix is still useful, however, in micro LC-FAB/MS. Coutant et al. described an interface that incorporates on-line UV detection and postcolumn matrix addition (N29) and used the glycopeptide antibiotic teicoplanin as a model system to demonstrate its usefulness. The interface was tested with both microbore and capillary LC columns, but the capillary column proved more mass sensitive and allowed spectra to be obtained at low picomole concentrations.

Interest in microcolumn LC detection was not restricted to mass spectrometry. In a series of articles, Synovec and Renn described a refractive index gradient (RIG) detector suitable for micro LC (N30–N32). The detector measures the radial refractive index gradient passing through a Z-configuration cell. With the addition of fiber optics, small volumes suitable for micro LC detection can be probed. A novel axial on-column detection approach was introduced by Xi and Yeung (N33). Absorption detection was accomplished by axial coupling of source light with 10–75- μm -i.d. capillary columns using an optical waveguide and special interface. In this system, the full length of the sample band is used, providing an increase in path length of up to 1000 times over conventional cross-beam arrangements. Finally, on-line coupling of LC microcolumns with a flame photometric detector (N34) and an ICP/AES system (N35) were described. The latter system included a low-consumption thermospray nebulizer.

Although applications are not normally the subject of Fundamental Reviews, several were noted that included significant method and/or technique developments of microcolumn LC. Micellar electrokinetic capillary LC of common drugs of abuse was described by Wernly and Thorman (N36). Several examples of ion-exchange separations in microcolumns were reported. Mueller et al. (N37) used 4.6- μm fused silica columns coated with (3-sulfopropyl)silane as a cation exchanger and (3-((2-aminoethyl)amino)propyl)silane as an anion exchanger to get fast separations (<35 s). Pfeffer and Yeung made capillary anion-exchange columns by first cross-linking vinylsilicone gums on the inside of fused silica capillaries and then dynamically modifying the stationary phase with cetyltrimethylammonium bromide (N38). Common anions were separated and then detected via indirect fluorescence detection. Ion-exchange separation of amino acids in open tubular columns after first derivatization with naphthalene-2,3-dicarboxaldehyde was reported for amino acids in bovine chymotrypsinogen (N39) and for amino acids in three individual neurons (N40).

O. TRACE ANALYSIS

Trace analysis via liquid chromatography inevitably involves sample enrichment, derivatization for sensitive and/or selective detection, or the use of a highly sensitive detector. Since fundamental developments in these areas have been discussed in other sections of this review (for example, sample enrichment using column switching techniques are reviewed in Section L), only a highly selective sampling of LC techniques used in trace analysis is presented here.

Coquart and Hennion described an on-line concentration, isolation and liquid chromatographic separation method for the analysis of trace organics in natural waters (O1). Concentration and isolation are accomplished with two precolumns connected in series; the first acts as a filter for removal of interferences, while the second actually concentrates target solutes. The technique is applicable even if no selective

sorbent is available for the specific analytes of interest. Detection limits of less than 0.1 ppb were achieved for polar herbicides in the chlorotriazine and phenylurea classes. A novel method for the determination of tetracyclines in animal tissues and fluids was developed, with sample extraction and cleanup based on the tendency of tetracyclines to chelate with divalent metal ions (O2). The metal chelate affinity precolumn was connected on-line to a reversed-phase HPLC column, and detection limits for several different tetracyclines in a variety of matrices were in the 10–50 ppb range. Braithwaite and Smith described an on-line enrichment and separation system for the analysis of the organochlorine pesticides heptachlor, dieldrin, DDT, and aldrin in water with the concentrator precolumn located across the sample loop connections of a standard LC injector (O3). The use of a high-performance cation-exchange column to concentrate and separate trace metals in water before inductively coupled plasma-mass spectroscopy detection was studied by Boomer et al. (O4). Vreuls and co-workers optimized the coupling of liquid chromatography with capillary gas chromatography for trace analysis (O5), focusing on the introduction of large amounts of polar solvents into the gas chromatograph. Coupled LC/capillary GC is one of the most powerful two-dimensional separation techniques available, but removal of the LC mobile phase without loss of peak integrity in the GC column can be difficult. In this study, a diphenyltetramethyldisilazane (DPTMDS)-deactivated retention gap was used with partially concurrent solvent evaporation. In addition, insertion of a splitter between the retention gap and the analytical column allowed considerable increase in the evaporation rate of solvent.

Two fundamental studies of particular importance for trace enrichment and analysis were noted. Guiochon and co-workers showed that the nonlinear displacement effect in displacement chromatography can result in compression of trace component bands and, in essence, sample enrichment (O6). Irth et al. studied adsorption-desorption of nitrogen heterocycles on metal-loaded thiol phases as a function of pH and metal ion (O7). Results indicated that the optimum pH for desorption could be predicted from the pK_1 of the N-heterocycle and the stability constant of the complex formed with the metal ion. Application of this material as a preconcentration phase was demonstrated with several barbiturates in plasma.

Other interesting applications of liquid chromatography in trace analysis included postcolumn derivatization of reducing saccharides for fluorescence detection (O8), separation and selective detection of alkyllead species (O9), determination of alkyl-, nitro-, and chlorophenols via precolumn derivatization with dansyl chloride and chemiluminescence detection (O10), speciation of trace metals using column switching (O11), and on-line minicolumn enrichment of trace metals as hexamethylenedithiocarbamate chelates (O12).

P. PHYSIOCHEMICAL MEASUREMENTS

Readers interested in this topic may want to consult the corresponding section in the Size Exclusion Chromatography Fundamental Review article of this issue for related studies.

Association/Ligand Binding Measurements

During this review period, there has been a substantial increase in the number of published reports on the use of HPLC to measure protein interactions with drugs or other ligands either in solution or immobilized as in the case of affinity chromatography. These areas have been reviewed by Sebille et al. (P1) and Nakagawa and Shibukawa (P2). Binding studies between proteins and lidocaine (P3), anticoagulants (P4, P5), amphetamines (P6), phenytoin (P7), aluminum (P8), and bilirubin (P9) have been published. HPLC was used to measure the partition between erythrocytes and a fluoroquinolone antibiotic (P10).

Cserhati et al. (P11) studied the interaction of benzodiazepine derivatives with amino acids and phospholipids using reversed-phase LC. Gilli et al. (P12) used an amino-bonded stationary phase as a model system for receptor interaction with benzodiazepines. A strong correlation was found between packing-benzodiazepine interaction and the receptor-benzodiazepine binding. Morris et al. (P13) evaluated the electrophilicity of DNA-binding benzodiazepines using HPLC.

Binding of RecA protein to double-stranded DNA was studied using ion-exchange LC (P14). Lectin-sugar binding constants were determined by HPLC and microequilibrium dialysis (P15).

Thuaud and co-workers (P16) investigated the drug-binding properties of β -cyclodextrin derivatives. The stability constants for inclusion complexes of β -cyclodextrin with sulfonylureas, sulfonamides, and *p*-aminobenzoic acid esters were reported by Wang et al. (P17). Arnold et al. (P18) found a direct correlation between the stability of β -cyclodextrin inclusion complexes of substituted aromatic compounds and HPLC retention times using bonded β -cyclodextrin packings. The complex formation constants between β -cyclodextrin and nitrophenol isomers were determined using HPLC (P19). Enthalpies and entropies of the complex formation were also obtained.

In addition to determining binding constants by injecting solutions of ligands and acceptors and determining the concentration of unbound species, one can also carry out these studies in which the ligand or acceptor is immobilized onto a support (affinity chromatography). Lee et al. (P20) investigated the effects of determining binding constants in affinity chromatography under nonlinear isotherm conditions. An approach called quantitative affinity chromatography, for characterizing high-affinity interactions was reported by Hogg et al. (P21) and Olson et al. (P22). This method allows quantitation of the binding of two components from the competitive effect of one component on the partitioning of the other component between an immobilized acceptor and solution at equilibrium. This procedure was used to determine the binding constants of heparin with antithrombin (P21, P22).

Hearn and co-workers (P23, P24) used affinity chromatography to study the interactions of proteins with triazine dye affinity sorbents. Domenici et al. (P25, P26) studied drug-protein interactions using a human serum albumin-based HPLC chiral stationary phase. A chemically bonded bovine serum albumin stationary phase was used by Lammers et al. (P27) to determine drug-protein binding interactions. Cummings et al. (P28) injected DNA onto an ion-exchange column to produce an intercalator affinity column. This column was then used to study the intercalation of anticancer drugs. Protein interactions with immobilized metal ions were investigated by Hutchens and Yip (P29) and Boyer et al. (P30).

A compilation of over 150 solute-micelle association constants was reported by Foley (P31). The interaction of benzene and naphthalene derivatives with micelles was investigated by Marina et al. (P32, P33). Solute-micelle interactions were also investigated by Kord et al. (P34) using LC. Although the study by Abu-Hamdiyyah and Kumari (P35) on partitioning properties of micelles does not involve LC, it should prove useful to those interested in this general field.

Conformational Studies

Hodges' group (P36) used reversed-phase HPLC to study model peptides that had a high potential of forming α -helical structures in a nonpolar environment. Based on retention time measurements, it was possible to predict the presence of α -helical structures. McLeod et al. (P37) used reversed-phase HPLC to investigate the solution conformation of insulin monomer. Their results suggested a partial unfolding of the insulin monomer. Karger and co-workers (P38) studied the unfolding of α -lactalbumin on weakly hydrophobic chromatographic surfaces using fluorescence measurements and LC.

Partition Coefficients

The determination of octanol-water partition coefficients ($\log P$) or hydrophobicity parameters using reversed-phase HPLC continues to be a useful approach for characterizing potentially biologically active compounds with regards to quantitative structure-activity relationships (QSAR). Because a linear relationship is usually observed between $\log P$ and \log of the capacity factor ($\log k'$), reversed-phase HPLC is a convenient method of estimating partition coefficients for QSAR studies.

Several reviews have appeared on the use of HPLC to determine partition coefficients and hydrophobicity of compounds using HPLC (P39-P41). In addition to conventional

reversed-phase columns for QSAR measurements, polybutadiene-coated alumina (P42), an octadecylpolyvinyl stationary phase (P43), and an octadecyl-derivatized poly(styrene-divinylbenzene) packing (P44) were evaluated. Warne et al. (P45) employed an octadecyl column in series with an amino stationary phase for QSAR studies. Lavine et al. (P46) used micellar LC for determining structure-activity relationships for a series of compounds.

QSAR studies using HPLC have been reported for benzenesulfonamides (P47), bile acids (P48), imidazoquinolone derivatives (P49), and nitrogen mustard derivatives (P50). Partition coefficient data from HPLC measurements were obtained on adenosine and xanthine derivatives (P51), β -adrenoceptor blocking agents (P52), 2-amino-2-oxazolines (P53), benzaldehyde derivatives (P54), benzodiazepines (P55), 2',3'-dideoxynucleoside analogues (P56), and pyrazine derivatives (P57).

Hou et al. (P58) determined the water solubility and octanol/water partition coefficient of a number of hydrophobic dyes using HPLC. Kaibara et al. (P59) evaluated the hydrophobic interaction between acidic drugs and bovine serum albumin using a hydrophobic parameter defined as the slope of the log-log plot of capacity factor versus reciprocal of methanol concentration in an aqueous mobile phase. Patel et al. (P60) used the partition coefficient as a parameter to describe solvent strength in reversed-phase HPLC. Using partition coefficients obtained by HPLC, Jenke (P61) studied drug-polymer interactions and applied these results to predict binding of these solutes to a poly(vinyl chloride) container. Hooijat et al. (P62) determined the partition coefficient of styrene and a styrene-divinylbenzene stationary phase at several temperatures as a function of mobile-phase composition.

Thermodynamic and Kinetic Studies

Vidal-Madjar and co-workers (P63, P64) investigated the adsorption kinetics of human serum albumin on a reversed-phase packing under mass-overload conditions. This study was done using the "split-peak" method whereby a fraction of the solute is eluted as a nonretained peak. Karger's group (P65) studied the adsorption-desorption isotherm hysteresis of β -lactoglobulin on a weakly hydrophobic stationary phase using frontal chromatography. Guiochon et al. (P66) measured the adsorption isotherms of D and L isomers of *N*-benzoylalanine on a bovine serum albumin stationary phase at different temperatures. From these data, enthalpy of adsorption and the isosteric heat of adsorption were obtained. Winzor et al. (P67) investigated the problem of determining kinetic parameters for the interaction of a solute with immobilized ligand sites in affinity chromatography.

Rheinlaender et al. (P68) studied the adsorption of oligopolyoxyethylene alkyl ethers from aqueous and nonaqueous solutions onto graphite using liquid-solid chromatography. Knox and Shibukawa (P69) used band broadening associated with the HPLC of an EDTA-chromium complex to estimate rate constants and activation energies for ligand substitution. Andersen and Birdi (P70) determined the enthalpy and entropy of adsorption of a homologous series of alkanes, alkylbenzenes, and polyaromatic hydrocarbons on a reversed-phase column. Pirkle and Readnor (P71) measured the enthalpy and entropy of adsorption of a series of homologous *N,N'*-bis(2,4-dinitrophenyl)- α,ω -diaminoalkanes on π -basic chiral stationary phases. The thermodynamics of complexation of alkylbenzenes with silver was determined with silica containing a silver stationary phase (P72). An HPLC approach was described for determining rate constants, energy barriers, and equilibrium constants for dynamic molecular processes (P73).

HPLC has been used quite extensively for determining reaction kinetics because of its ability to separate products and reactants and ease of quantitation. Selected examples published during this review period include the determination of enzyme kinetics (P74-P80), hydrolysis kinetics (P81-P85), and degradation/stability kinetics of drugs (P86-P89), retinol (P90), and prostaglandins (P91).

The effect of solvents on the isomerization and equilibrium kinetics of β -carotene were monitored by HPLC and a diode-array detector (P92). Racemization half-lives of enantiomeric oxazepam were determined with a spectropolarim-

eter immediately following separation on a chiral stationary phase (P93). In addition, rates of hydrolysis of racemic and enantiomeric 3-*O*-acyloxazepans by esterases were measured. The kinetics of interconversion of conformational diastereomers of 2,3-benzodiazepines were studied using HPLC (P94). Mannschreck and Kiessl (P95) followed the kinetics of enantiomerization of a chiral helicene hydrocarbon by deconvoluting chromatograms using photometric and polarimetric detection.

The hydrolysis kinetics of iodine to iodide were determined by Rahn (P96) using reversed-phase HPLC with an ion-pairing mobile phase and electrochemical and UV detection. Chen et al. (P97) reported on an equilibrium and kinetic study of ligand exchange reactions between N,N-disubstituted dithiocarbamate chelates of nickel(II).

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